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THE IMMUNOLOGICAL ANTIGENICITY OF RAPID HEAT INACTIVATED VIRUSES I. NEWCASTLE DISEASE VIRUS*

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A HEAT exchanger has been developed at the University of Massachusetts for studying the effect of high temperatures for short periods of time on pathogenic bacteria which may be found in milk and milk products.[2,4,5] This machine is so constructed that it is possible to obtain any temperature up to 230 F in a fraction of a second and to heat for times as short as 0.033 second. In high temperature short-time studies on *Salmonella typhimurium*,[1] it was found that antigenicity was affected much less by heating to an inactivation temperature of 194.9 F in 0.033 second than by inactivation by heating at 140 F for one hour. Thus, the nature of this heating instrument has made it potentially possible to inactivate a specific pathogenic agent with a theoretical minimum of destructive effect upon its antigenic structure.

It was desired to study the effect of this heat inactivation principle on viruses for the purpose of learning whether the rapid heat treatment method is applicable for the preparation of inactivated virus vaccines. The virus of Newcastle disease (NDV) was selected for the initial investigation because of its easy cultivation and high infective titer for the chicken embryo, and the simplicity of serological testing and accessibility of chickens for immunization procedures.

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* Contribution No. 1193 from the University of Massachusetts College of Agriculture Experiment Station, Amherst, Massachusetts.

MATERIALS AND METHODS

The heat exchanger used in this investigation consists of a stainless steel pressure tank or reservoir and small bore stainless steel tube. The tube is heated by high amperage, low voltage alternating current. The suspension of the agent to be treated is forced through the small bore tube by air pressure at a rate so as to insure turbulent flow as determined by the Reynold's number,[6] and is heated by heat exchange from the small bore tube. This machine is further described by Read, Boyd, Litsky and Hankinson.[3]

The procedure for the heating and collection of samples was as follows: a given pool of NDV-infected allantoic fluid was thawed, and titrations of both hemagglutinin and infectivity were made. A 1:10 dilution of the virus was then prepared with sterile 0.85 percent sodium chloride so as to give a final volume of 2 to 3 liters. This material was then introduced into the sterile reservoir of the heat exchanger, and compressed air was applied to initiate controlled flow of the fluid. The fluid was heated in 0.033 second to the highest temperature level to be tested and desired final heating temperatures were regulated at different intervals. Heated samples were collected in 500 ml Ehrlenmeyer flasks containing glass beads of five-eighths inch diameter previously cooled to –25 C. The beads served as a cooling mechanism. Samples taken were usually of 20 to 30 mls in volume. There was an inherent holding time of 0.022 second due to the sample collecting technique.

The 9251 strain of NDV was used in this investigation, and had been passaged 125 times in embryonating chicken eggs at the time of receipt, and was passaged three more times prior to testing. This particular NDV strain 9251 has been characterized as a mesogenic type virus possessing strong virulence for embryonated eggs and capable of causing clinical Newcastle disease in infected adult birds. Pools of allantoic fluid containing virus were prepared from 10 or 11 day-old embryonating eggs which had been inoculated with a 1:10 or 1:100 dilution of infective allantoic fluid. The pools of virus were frozen and stored at –25 C prior to use. Both hemagglutination (HA) and egg infectivity titrations EID_{50}) were performed on each pool after thawing and prior to testing. Fresh

chicken red blood cells for hemagglutination titrations were obtained daily and washed with 0.85 percent sodium chloride. A one percent suspension of cells was used in the titrations and a five percent suspension for spot testing allantoic fluid for hemagglutination in the virus infectivity studies. All birds used in this investigation were tested serologically prior to use. Vaccinated and young developing chickens were held in the same quarters. The unvaccinated birds also served as controls for the vaccinated ones, since they were bled prior to vaccination and their sera examined for antibody against Newcastle disease by both the hemagglutination inhibition (HI) (alpha method) and the serum neutralization test (SN) in embryonating chicken eggs.

The testing procedure was as follows: each sample, representing the attainment of a different temperature level in 0.033 second time, was tested for its hemagglutinability and infectivity for 10-day-old embryonating chicken eggs. The sample representing the minimum temperature demonstrating no infectivity was further tested for viral infectivity by means of three blind passages of allantoic fluid in embryonating eggs. Upon confirmation of the absence of viral infectivity of the sample, it was used to vaccinate NVD susceptible 4-week-old chickens.

RESULTS AND DISCUSSION

The first vaccine batch to be tested (batch 1) had an

TABLE I

Inactivation of hemagglutination and infectivity by heating batch 1 to various temperatures in 0.033 second

Temperature virus heated to in 0.033 second	Highest dilution giving hemagglutination	Infectivity for embryonated eggs
Unheated	1:16	4/4
135° (pre-heat)	1:16	4/4
170	1:8	4/4
171	1:4	4/4
172	1:2	4/4
173	1:2	4/4
174	negative	4/4
175	"	3/4
176	"	2/4
177	"	4/4
178	"	2/4
179	"	negative
180	"	"

Note: All eggs for infectivity studies were inoculated with 0.1 ml each of the specified sample.

EID_{50} titer of 1×10^{12} per ml. As can be seen from table 1, HA was completely lost at 174 F, and at 179 F the ability to infect embryonating eggs was also lacking. Virus infectivity determinations on the 179 F sample were found to be negative.

Four-week-old chicks were vaccinated intramuscularly with 0.5 ml of the 179 F sample every 7 days for 3 weeks. The vaccinated birds were bled one, 2, 10 and 19 weeks after the final vaccination, and their sera examined for the ability to inhibit hemagglutination and to neutralize virus (table 2). Four of the five birds tested demonstrated a strong immunological response by the SN test through the second week following the final vaccination with titers ranging from 3×10^{10} to 4×10^{8} per ml. The SN titers for the 10 week period were lower than for the 2 week period and ranged from 2×10^{8} to 4×10^{3} per ml. At 19 weeks following the final vaccination, the titers had dropped slightly but neutralization of virus was still measurable. The HI titers at the 19 week period were rather erratic, however. Three birds remained consistently strong through the 10 week period. The HI titers at 19 weeks ranged from 1:640 to 1:10.

It was desirable to show that the foregoing could be successfully reproduced. Therefore a second batch (batch 2) was prepared in a like manner. The EID_{50} prior to heating was 1×10^{11} per ml. Final heating temperatures ranged from 172 F to 176 F. Table 3 shows that HA was lost at 174 F and that the 175 F sample was unable to infect embryonating eggs. Virus infectivity studies on the 175 F sample were all found to be negative.

An evaluation of the immunological potential of the 175 F sample was made. Fifteen birds were divided into three groups. Group 1 received one inoculation of 1.0 ml, group 2 received two 1.0 ml inoculations spaced 3 days apart, and group 3 received three inoculations spaced 3 days apart. All inoculations were by the intramuscular route. The birds in the one and two inoculations groups were bled at 2, 3, 6 and 15 weeks after vaccination; and the birds in the three inoculations group were bled 2, 6 and 15 weeks after the final vaccination. All sera were tested for the ability to inhibit hemagglutination and to neutralize virus. The results are presented in table 4.

TABLE II

Hemagglutination inhibition and serum neutralization results for sera from NDV susceptible chickens vaccinated with 0.5 ml of the 179 F inactivated sample of batch 1

Bird No.	Weeks after final vaccination: 1 week HI*	1 week SN**/ml	2 weeks HI	2 weeks SN/ml	10 weeks HI	10 weeks SN/ml	19 weeks HI	19 weeks SN/ml
6	>1:1280	6×10^{10}	>1:20,480	3×10^{10}	>1:320	7×10^{4}	1:320	$>5 \times 10^{3}$
7	>1:1280	2×10^{10}	>1:20,480	3×10^{10}	>1:320	1×10^{5}	1:320	$>5 \times 10^{3}$
8	>1:1280	1×10^{6}	>1:20,480	3×10^{10}	>1:320	4×10^{3}	1:160	5×10^{2}
9	1:80	1×10^{6}	1:80	8×10^{8}	1:20	4×10^{4}	1:640	$>5 \times 10^{3}$
10	>1:1280	1×10^{11}	>1:20,480	4×10^{8}	1:40	2×10^{3}	1:10	5×10^{2}
Control		negative	1:10	negative	1:10	negative	1:10	negative

* HI Hemagglutination inhibition
** SN Serum neutralization

Note: Pooled sera from five control birds comprise the control sample tested.

TABLE III

Inactivation of hemagglutination and infectivity by heating batch 2 to various temperatures in 0.033 second

Temperature virus heated to in 0.033 second	Highest dilution giving hemagglutination	Infectivity for embryonated eggs
Unheated	1:64	4/4
135° F (pre-heat)	1:64	4/4
172	1:64	3/4
173	1:64	3/4
174	negative	1/4
175	"	0/4

Note: All eggs for infectivity studies were inoculated with 0.1 ml each of the specified sample.

On the basis of the relatively short term, post-vaccination period being reported here, a substantial immune response appeared with birds no. 13 and 14, 2 weeks after one vaccination. Three birds (19, 20, and 23) of the two inoculations group, for the same time interval, yielded hemagglutination inhibition titers of 1:2560 or more and all five birds (19, 20, 21, 22, and 23) had serum neutralization titers ranging from 1 x 10^{12} to 3 x 10^{8} per ml at this period. At the 3 week period, all birds in the one and two inoculations groups had generally consistent serum neutralization titers ranging from greater than 3 x 10^{10} to 1 x 10^{7} per ml. At the six week period SN titers ranged from greater than 2 x 10^{5} to less than 2 x 10^{2} per ml for all three groups. The HI titers at the 3 week period for the one inoculation group and the 2 week period for the second and third groups were fairly consistent, at greater than 1:640, with the exception of bird no. 27 of the three inoculations group yielding a 1:80 HI titer.

At 15 weeks, four birds in the one inoculation group still yielded substantial HI titers which ranged from greater than 1:2580 to 1:320. The second and third groups yielded essentially similar results. The SN results appeared to be fairly consistent for all three groups with titers ranging from greater

TABLE IV

Hemagglutination inhibition and serum neutralization results for sera from NDV susceptible chickens vaccinated with 1.0 ml of the 175 F sample of batch 2

Bird No.	Weeks after final vaccination: 2 weeks HI	2 weeks SN/ml	3 weeks HI	3 weeks SN/ml	6 weeks HI	6 weeks SN/ml	15 weeks HI	15 weeks SN/ml
(one inoculation)								
13	>1:2560	3×10^8	>1:640	1×10^{10}	>1:320	1×10^5	1:640	$>5 \times 10^3$
14	>1:2560	5×10^5	>1:640	1×10^7	1:40	$<2 \times 10^2$	1:10	5×10^1
16	1:40	3×10^3	>1:640	5×10^7	>1:320	$>2 \times 10^5$	1:640	$>5 \times 10^3$
17	1:40	$>1 \times 10^3$	>1:640	$>3 \times 10^{10}$	>1:320	5×10^3	1:320	8×10^2
18	1:20	6×10^4	>1:640	3×10^8	>1:320	$>2 \times 10^4$	>1:2560	$>5 \times 10^3$
(two inoculations)								
19	1:2560	1×10^{10}	>1:640	$>3 \times 10^{10}$	>1:320	1×10^5	>1:640	$>5 \times 10^3$
20	1:2560	1×10^{12}	>1:640	$>3 \times 10^{10}$	1:320	$>2 \times 10^5$	>1:640	$>5 \times 10^3$
21	1:80	1×10^8	>1:640	$>3 \times 10^{10}$	>1:320	6×10^3	1:160	2×10^2
22	1:40	3×10^8	>1:640	4×10^9	>1:320	1×10^5	>1:640	5×10^2
23	>1:2560	3×10^8	>1:640	$>3 \times 10^{10}$	>1:320	$>2 \times 10^5$	>1:640	7×10^3
(three inoculations)								
24	>1:640	$>3 \times 10^{10}$			>1:320	$>2 \times 10^5$	—*	
25	>1:640	$>3 \times 10^{10}$			>1:320	1×10^5	—*	
26	>1:640	$>3 \times 10^{10}$			>1:320	1×10^5	>1:640	$>1 \times 10^4$
27	1:80	4×10^9			1:160	1×10^3	1:320	2×10^2
28	>1:640	$>3 \times 10^{10}$			>1:320	1×10^5	>1:640	5×10^3

* Birds died during the 15 week period. No evidence of NDV.

Note: Pooled sera from five control birds were tested at different intervals and no inhibition of hemagglutination or neutralization of test virus was evident at any time.

than 5×10^3 to 2×10^2 per ml with the exception of one bird which apparently did not respond.

The results from this experiment seem to indicate that the number of inoculations made little or no difference on the strength of the immunological responses obtained, since one inoculation produced essentially the same effect as two or three.

Batch 2 vaccine had been stored at –25 C from the time of preparation to 4½ months when its antigenic potency was re-evaluated. Since the birds originally inoculated with batch 2 had received 1.0 ml each, a reduced vaccination volume test accompanied the storage potency test of the vaccine. Twenty four-week-old chicks were vaccinated intramuscularly with 0.5 ml of the stored batch 2. The birds were bled 4½ weeks after their vaccination and sera tested for the ability to inhibit hemagglutination and to neutralize virus. The HI and SN titers for this experiment are given in table 5. Eleven of the 20 birds yielded HI titers of greater than 1:2560, and one at 1:1280, one at 1:320, and two at 1:160. The remaining five birds responded with HI titers of less than 1:160. Thirteen of the vaccinated birds yielded SN titers ranging from greater than 3×10^9 to 1×10^7. The remaining seven birds had titers of less than 3×10^5. Thus, 75 percent of the birds in this experiment yielded significant responses based on the HI tests above. It is important to emphasize that the dosage was 0.5 ml per bird and that only one vaccination was used, and also that the vaccine preparation had been stored for four and one-half months at –25 C.

All of the vaccinated birds, thus far described, were challenged along with contact controls and additional susceptible and immune controls with a virulent strain of Newcastle disease virus. The contact control birds had been hatched and raised in close proximity with the vaccinated birds and were bled and serologically tested to re-establish their individual susceptibilities to Newcastle disease as were all of the vaccinated and additional test controls (immune and susceptible).

The test immune control chickens were furnished by the

TABLE V

Hemagglutination inhibition results for sera from NDV susceptible chickens vaccinated with 0.5 ml of the 175 F sample of batch 2 after storage at freezer temperature for 4½ months, (–25 C).

Bird No.	HI titer	Four and one-half weeks after vaccination (SN titer per ml)
36	1:1280	$<3 \times 10^5$
37	>1:2560	$>1 \times 10^9$
39	>1:2560	$>2 \times 10^9$
40	<1:10	5×10^2
41	>1:2560	6×10^7
42	1:40	6×10^8
43	>1:2560	$>3 \times 10^9$
44	1:320	1×10^1
45	1:80	3×10^1
46	>1:2560	$>3 \times 10^9$
47	<1:10	4×10^1
48	>1:2560	1×10^7
49	>1:2560	$<3 \times 10^5$
50	>1:2560	$>3 \times 10^9$
51	1:160	5×10^8
52	1:160	$<3 \times 10^5$
53	1:10	2×10^8
54	>1:2560	$>3 \times 10^9$
55	>1:2560	$>3 \times 10^9$
56	>1:2560	2×10^8
Control	1:10	negative

Note: Pooled sera from five control birds comprised the control sample.

Department of Veterinary Science at the University of Massachusetts and were hatched and reared in the laboratory. They were immunized for Newcastle disease in an isolation unit. The age of the birds at the time of challenge was approximately 5 months.

The test susceptible control chickens were from two sources, Plymouth Rock and sex-link. The former were laboratory hatched and reared in isolation units. They were approximately 3½ to 4 months old at the time of challenge. The latter were mature laying hens approximately 15 months of age. These birds were hatched and reared at the laboratory and placed on a nearby farm. Some of these birds had not been

vaccinated for Newcastle disease prior to the time they were delivered to the farm.

The challenge Newcastle disease virus was prepared from a culture obtained from Dr. Robert P. Hanson, Department of Veterinary Science, University of Wisconsin, from the Newcastle disease virus repository, and identified as Texas GB 1948. It was passed in embryonated eggs and the harvested allantoic fluid was used as the challenge virus. The allantoic fluid suspension had a 2.5 x 10^9/0.1 ml chick embryo LD_{50} titer. It was then diluted so as to give approximately 40,000 chick embryo infective doses in 0.5 ml. A challenge dose of 0.5 ml was given to each bird intra-muscularly in the pectoral region.

The vaccinated birds were challenged in groups already established for the vaccination portion of the investigation. Group 1, consisting of birds 6 to 10 which had received three 0.5 ml inoculations at seven day intervals, and group 2 consisting of birds 13 to 18 which had received a single 1.0 ml inoculation were challenged approximately 7 months after vaccination. Group 3 consisting of birds 19 to 23 which had received two 1.0 ml vaccinations spaced 3 days apart and group 4 consisting of birds 24 to 28 which had received three 1.0 ml vaccinations at intervals of 3 or 4 days were challenged approximately six months after vaccination. Group 5 consisting of birds 36 to 56 had received one 0.5 ml inoculation of the "Rapid Heat" inactivated virus which had been stored at –25 C for approximately 4½ months and were challenged approximately 4 months later. Group 6 consisted of twelve contact susceptible control birds, Group 7 consisted of thirteen susceptible test control birds and Group 8 consisted of 8 immune test control birds.

It should be noted here that none of the birds vaccinated with the "Rapid Heat" inactivated Newcastle disease virus had been immunized against infectious bronchitis. This was an oversight on the part of the investigators. Consequently, the majority of the vaccinated and susceptible contact control birds contracted infectious bronchitis during the challenge period as evidenced by both clinical signs and necropsy findings.

The results of this challenge experiment are summarized in table 6. Only one of the thirty-seven vaccinated and challenged birds died after challenge. Three of the 12 contact susceptible controls and seven of the 13 susceptible test controls died. None of the immune controls died or gave any indication of illness. Six of the vaccinated birds gave either questionable or positive indications of Newcastle disease susceptibility by either demonstrating signs of illness or by the finding of lesions at necropsy while the remaining thirty-one were definitely shown to be immune to challenge.

HI titers prior to challenge are given as the geometric means for the participating subjects. The geometric mean HI titer for group 6 was 1076 and this was the highest of all of the vaccinated groups. Second was group 5 with a titer of 816 and third group 3 with 761.

Thus, 84 percent of the vaccinated birds were found to be immune to Newcastle disease virus while all of the unvaccinated susceptible controls were found to be susceptible.

The results of this investigation apparently validate the concept that by raising the temperature of inactivation, and shortening the exposure time, there is a minimum of destructive denaturation of vital proteins comprising the antigenic nature of the particular agent.

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SUMMARY

A method has been developed utilizing the principle of heating to relatively high temperatures in short periods of time to inactivate the virus of Newcastle disease. This resulted in a product of apparently high immunological antigenicity.

TABLE VI

Challenge results for all birds vaccinated with "Rapid Heat" inactivated Newcastle disease virus

Group number	Number of birds	Number of vaccinations	Months after vaccination	Geometric mean HI Titer before Chall.	Mortality no. dead/total	Signs	Lesions	NDV Status no. immune/total
I	4	3	7	269	0/4	0/4	0/4	4/4
II	5	1	7	422	0/5	0/5	0/5	5/5
III	5	2	6	761	0/5	0/5	1/5	4/5
IV	4	3	6	1076	1/4	3/4	1/4	1/4
V	19	1	4	816	0/19	0/19	2/19	17/19
VI	12	unvaccinated	—	8	3/12	9/12	3/12	0/12
VII	13	"	—	5	7/13	7/13	3/13	0/13
VIII*	8	1	3	1973	0/8	0/8	0/8	8/8

* Wing web vaccination with active NDV-9251.

Newcastle disease virus-susceptible four-week-old chickens were vaccinated with the thermal inactivated virus suspensions and comparable immune responses were obtained with single as well as multiple vaccination and dose volumes ranging from 1.0 to 0.5 ml. Substantial immune responses were obtained in birds up to 19 weeks following vaccination.

Immunizing potency of the virus suspension was apparently retained following a four and one-half months storage period at –25 C, since birds vaccinated with a 0.5 ml volume of this material responded immunologically when tested by the hemagglutination inhibition and serum neutralization tests.

Eighty-four percent of all of the birds which were challenged from 4 to 7 months after vaccinations were found to be immune to Newcastle disease, while 100 percent of the unvaccinated controls were shown to be susceptible.

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THE USE OF FURAZOLIDONE FOR CHUKAR PARTRIDGES* †

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FOR a number of years, a heavy mortality of baby chukar partridges (*Alectoris graeca*) has been experienced at the New Mexico State Game Bird Farm during the latter part of the hatching season. Mortality has been particularly severe during the first 4 weeks after hatching.[2] *Salmonella derby* has been isolated from a number of dead chukars from the game farm.[3]

Jerstad[5] reported that 0.011 percent furazolidone (FZD) gave good results in controlling field infection of blackhead in chukar partridges. Unfortunately, there were no details given. It has been reported[1] that game birds may be treated with 0.011 to 0.022 percent FZD for 7 to 10 days followed by 0.0055 percent FZD for another 7 to 10 days. The only game birds specifically mentioned were quail and pheasants. It was suggested that the turkey programs be followed for game birds. Recent reviews[3,4,6,7] of the uses of FZD indicate that it may be effective in reducing mortality or stimulating growth in a number of avian species. Yet no data were given for chukar partridges.

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A report of results obtained from feeding 0.011 percent FZD to day-old chukar partridges will aid in filling a void concerning the use of this drug.

MATERIALS AND METHODS

The experiments reported herein were undertaken at the New Mexico State Game Bird Farm and were conducted under commercial conditions. Three hatches of chukars were made beginning July 7, 1959. This was the end of the hatching season. Each hatch was divided at random into a treated or control group. Groups were further divided when more than 100 chukars were in each original group. The baby chukars were placed in clean, disinfected brooder houses on sugarcane litter. A commercial feed and water were available *ad libitum*. The treated groups were fed the commercial ration containing 0.011 percent FZD from one day of age to 8 weeks. The drug was mixed in the ration by the manufacturer. A sample of 30 birds from each pen of hatches 9 and 10 was weighed when they were 4 and 3 weeks of age respectively. All birds that died were recorded according to pen number. A sample of the dead birds was immediately frozen and sent to the poultry pathology laboratory at the New Mexico State University for examination. Standard procedures were used for attempted isolation of Salmonella or other pathogenic bacteria. Two lots of eggs consisting of 72 and 115 eggs, which were infertile eggs or dead embryos from completed hatches, were examined for Salmonella.

RESULTS

The results obtained for growth of the chukar partridges are given in tables 1 and 2. The chukars fed 0.011 percent FZD were significantly heavier than the controls at both three and four weeks of age. The coefficient of variability was much larger in the controls of hatch 9 than for hatch 10.

Mortality occurring in the three lots to 48 days of age is given in table 3. There was a highly significant difference in mortality between means of the control and treated groups of lot 9.

TABLE I

Mean body weight and coefficient of variation of chukar partridges fed 0.011 percent FZD from 0 to 4 weeks or 0 to three weeks and their control groups

Pen	Treatment	Lot 9 (4 wks.) Wt. gms.	CV	Lot 10 (3 wks.) Wt. gms.	CV
C	Control	104 ± 3.42[1]	18.7	81 ± 2.5	16.9
D	Control	117 ± 4.83	22.6	82 ± 2.7	17.8
Mean		110 ± 3.28	23.1	81 ± 1.8	17.4
A	0.011% FZD	144 ± 2.87	15.7	98 ± 2.9	15.9
B	0.011% FZD	142 ± 2.13	8.2	102 ± 2.7	14.4
Mean		143 ± 2.33	12.6	100 ± 1.7	13.1

[1] Mean ± standard error.

TABLE II

Analysis of variance of body weight of chukar partridges at 4 and 3 weeks of age

Source	DF	Lot 9 MS	Lot 10 MS
Total	119		
Treatments	1	32341**	10622**
Pens treated alike	2	1332	132
Birds within pens	116	484	199

** Highly significant.

Salmonella anatum was isolated from four dead birds from the control groups of hatch 9. Examination of the dead embryos or infertile eggs received after the hatches were completed was negative.

DISCUSSION

The presence of *Salmonella derby* in chukars at the game farm in 1957 led to the suggestion that 0.011 percent FZD be fed to the chukars immediately after hatching for at least 4 weeks. The improvement in growth of the chukars fed FZD at 0.011 percent in the ration as determined by body weight was highly significant whether measured at 3 or at 4 weeks of age. The coefficient of variability was also reduced in the treated groups.

There was a highly significant reduction in mortality for the treated groups when compared with the controls at 48 days of age. The greatest effect was noted in hatches 9

TABLE III

Mortality to 48 days of age in chukar partridges fed a ration with and without 0.011 percent furazolidone

Pen	Treatment	Hatch Number 9 Dead	9 Percent	10 Dead	10 Percent	11 Dead	11 Percent	Total Dead	Total Percent
C	Control	33/93[1]	35.5	10/56	17.9	26/90	28.9	69/239	28.9
D	Control	16/93	17.2	3/56	5.4			19/149	12.8
	Total	49/186	26.3	13/112	11.6	26/90	28.9	88/388	22.7
A	0.011% FZD	13/93	13.9	2/156	3.6	16/90	17.8	31/239	12.9
B	0.011% FZD	9/93	9.7	2/56	1.8			10/149	6.7
	Total	22/186	11.8	3/112	2.7	16/90	17.8	41/388	10.6
	P-Value		3.56**		2.59*		1.59		4.43**

[1] Number dead/number started.
* Difference between pooled value of control and treated groups. Significant at five percent level.
** Difference between pooled value of control and treated groups. Significant at one percent level.

and 10. The high mortality of 80 percent experienced in the past years was not present in 1959.

It is rather interesting that the Salmonella isolated was not the same one isolated two years ago. This is hard to explain, as new breeder stock is selected from the previous year's hatch prior to release of the remaining chukars in the field. There is a possibility that selection is made from the oldest birds. This could eliminate many birds that would remain carriers. The fact that no Salmonella was isolated from the eggs or dead embryos suggests some other source of infection of *Salmonella anatum*. Extreme care is taken to insure that there are no hiding places for vermin. However, sparrows, as well as other wild birds, are frequent visitors of the farm, and these may serve as a source of infection. In spite of the reduction in mortality, it was impossible to determine whether carriers resulted.[4] This area bears further investigation.

FZD was fed to all birds for at least 8 weeks without any outward signs of any toxic effects. This was also evident by the improvement in growth that was observed for the first 4 weeks.

ACKNOWLEDGMENT

Acknowledgment is made to Dr. P. R. Edwards, in whose laboratory at the United States Public Health Service, Communicable Disease Center, Chamblee, Georgia, these cultures were typed.

SUMMARY

There was a significant reduction in mortality from a naturally occurring infection of *Salmonella anatum* in day-old chukar partridges when 0.011 percent FZD was fed beginning immediately after hatching.

There was a significant increase in body weight of chukar partridges fed 0.011 percent FZD to either 3 or 4 weeks of age.

Visible signs of toxicity to the drug were not observed when the drug was fed for eight weeks.

The isolation of *Salmonella derby* in 1957 and *Salmonella anatum* in 1959 from chukar partridges reared at a game farm in New Mexico is reported.

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THE EFFECT OF FURAZOLIDONE ON *SALMONELLA PULLORUM* AND AGGLUTINATION TITERS IN CHICKENS

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MANY investigators have studied the effect of furazolidone on *Salmonella pullorum* infection. Smyser and

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* N-(5-nitro-2-furfurylidene) -3-amino-2-oxazolidone supplied as 11 percent mixture (nf 180) through the courtesy of Hess and Clark, Inc., Ashland, Ohio.

Van Roekel[5] and Henderson *et al.*[3] have reviewed this work. The purpose of this report is to present more information on the effect of furazolidone on the control of *S. pullorum*.

MATERIALS AND METHODS

In the first experiment each of 36 14-week-old Single Comb White Leghorn chickens was inoculated orally with 2 ml of a 48-hour broth culture of the same isolate of *S. pullorum* used in our previous report.[3] At this time all birds were negative to the tube agglutination test at one to ten dilution with a pullorum antigen made with strains 17, 19 and 20. All birds were fed an all-purpose ration without any medication other than .011 percent furazolidone.* After 5 weeks on the drug, the surviving birds were divided into two equal groups and one group was given the ration without furazolidone.

In the second experiment, a group of 53 9-week-old, Vantress-Arbor Acre cross chickens were inoculated orally as in the first experiment. All birds were negative to the pullorum tube agglutination test. In this experiment, ten birds received the same ration without furazolidone from the start of the experiment. As before, after 5 weeks, the group receiving furazolidone was divided and half the birds were given the nonmedicated ration.

In both experiments, individual pullorum agglutination titers were determined each week except on the 20th and 23rd weeks of the first experiment. Two and one-half weeks after inoculation and weekly thereafter, with the exception of the 20th and 23rd weeks in experiment one, four birds were killed and cultured for *S. pullorum*. A piece of each of the following organs was put into separate tubes of nutrient broth (Difco) and selenite (Difco) : heart and pericardium, liver, gall bladder, spleen, pancreas, gonads, kidneys, ceca with adjacent intestine, and crop. These were incubated at 37 C for 16 hours and subcultures were made on brilliant green agar (Difco) and tryptose agar (Difco) plates. The broth cultures were incubated at room temperature for another 24 hours and again transfers were made to the two solid media. After incubation at 37 C for 24 hours the plates were held at room temperature

for a maximum of 10 days. Whenever colonies typical of *S. pullorum* developed, they were transferred to carbohydrate broths and other appropriate media for the identification of *S. pullorum*. To determine the antigenic forms present, isolates from each bird were typed by the macroscopic sedimentation test described by Williams.[7]

RESULTS

The results of experiment 1 are given in table 1. During the 12 weeks when the birds were receiving furazolidone, no detectable pullorum agglutination titer was obtained. Twenty-six birds were cultured while they were receiving furazolidone. *S. pullorum* was recovered from one of these birds which was negative to the tube test. This isolate was typed as a standard strain with the method described by Williams.[7] It is of interest to note that this recovery was made after the bird had been receiving furazolidone for 12 weeks.

TABLE I

The effect of furazolidone on titer and recoverability of *S. pullorum* inoculated into 14-week-old S.C.W. Leghorn chickens

Age in weeks	.011 percent furazolidone in feed			
	Serum agglutination		Isolation of *S. pullorum*[2]	
16	0/36[1]		0/4[1]	
17	0/32		0/4	
18	0/28		0/4	
19	0/24		0/4	
21	0/10	0/10[3]	0/2	0/2[3]
22	0/8	1/8 (1/100)[4]	0/2	0/2
24	0/6	1/6 (1/250)[4]	0/2	1/2[4]
25	0/4	1/4 (1/25)[5]	0/2	0/2
26	0/2	1/2 (1/25)[5]	1/2	1/2[5]

[1] Number positive over number tested or cultured
[2] Bacteriological media were inoculated with materials from chickens whose serum had been tested for agglutinins four days previously.
[3] At 19 weeks, after 5 weeks on the drug, the birds were divided into two lots of ten birds. This ten did not receive furazolidone for the remainder of the experiment.
[4] Bird number 320—positive titer in brackets.
[5] Bird number 309—positive titer in brackets.

In the 10 birds taken off furazolidone, one bird developed a *S. pullorum* titer 3 weeks after removal of the drug. Another bird developed a titer 6 weeks after removal of furazolidone.

S. pullorum was recovered from each of these birds. The other eight birds on the ration without furazolidone did not develop detectable titers, and the organism was not recovered.

The results of experiment 2 are given in table 2. In order to establish an infectivity rate, ten untreated birds were infected with *S. pullorum*. Nine of these birds developed an agglutination titer and *S. pullorum* was recovered from six of the ten birds. Of the birds which received furazolidone continuously, two developed a detectable agglutination titer, 4 and 6 weeks after infection. *S. pullorum* was recovered from the bird which was positive at the 1/100 dilution, but not from the one whic hgave a positive titer at the 1/25 dilution. Three weeks after infection, *S. pullorum*, again a standard strain, was recovered from one treated bird which did not develop a detectable agglutination titer. In the group of birds which

TABLE II

The effect of furazolidone on titer and recoverability of *S. pullorum* inoculated into 9-week-old Vantress-Arbor Acre cross chickens

Age in weeks	.011 Furazolidone in Feed				Untreated Infected Controls	
	Serum agglutination		Isolation of *S. pullorum*[2]		Serum agglutination	Isolation of *S. pullorum*[2]
11	0/43[1]		0/2[1]		7/10	1/2
12	0/41		1/2		8/8	2/2
13	1/39(1/50)[4]		0/2		6/6	1/2
14	1/37(1/100)[4]		1/2[4]		4/4	2/2
15	1/19(1/10)[5]	0/18[3]	0/2	[3]	1/2	0/2
16	1/17(1/10)[5]	0/18	0/2	0/2		
17	1/15(1/25)[5]	1/16(1/100)[6]	0/2	1/2[6]		
18	1/13(1/25)[5]	0/14	0/2	0/2		
19	1/11(1/10)[5]	0/12	0/2[5]	0/2		
20	0/9	0/10	0/2	0/2		
21	0/7	0/8	0/2	0/2		
22	0/5	0/6	0/2	0/2		
23	0/3	0/4	0/2	0/2		
24	0/1	0/2	0/1	0/2		

[1] Number positive over number tested or cultured

[2] Bacteriological media were inoculated with materials from chickens whose serum had been tested for agglutinins 4 days previously.

[3] At 14 weeks, after 5 weeks on the drug, the birds were divided into two lots of 19 and 18 birds. The 18 did not receive furazolidone for the remainder of the experiment.

[4] Bird number 71—positive titer in brackets.

[5] Bird number 21—positive titer in brackets.

[6] Bird number 18—positive titer in brackets.

received furazolidone for 5 weeks and then taken off medication, one bird became positive to the tube agglutination test 3 weeks after removal of the drug, and *S. pullorum* was isolated from this bird. The other 17 birds did not develop detectable agglutination titers, and *S. pullorum* was not isolated.

In both experiments, 3 weeks after the removal of the drug, one bird developed an antibody titer high enough to designate it as a positive reactor to the tube agglutination test. *S. pullorum* was isolated from both of these birds. In the first experiment, 6 weeks after the removal of the drug, one bird was positive to the tube test at the one to twenty-five dilution and *S. pullorum* was recovered.

DISCUSSION

In a previous report[3] furazolidone at .011 percent of the ration did not materially affect the agglutination titers of adult chickens artificially infected with *S. pullorum*, except perhaps during the bacteremia. In the current test, two birds developed a titer while on the drug. One of these reached a positive titer at one to one hundred, and *S. pullorum* was recovered. The other bird reacted at the one to ten dilution, increased to one to twenty-five, and returned to the one to ten dilution. The organism was not recovered from this bird. This seems to indicate that sufficient organisms developed to stimulate enough antibodies at one to twenty-five dilution, but that the drug was able to reduce the number of organisms to lower the titer and reduce the chances of recovering the organism.

Newman and Krueger[4] have suggested that the *S. pullorum* organism may be confined to "walled off" foci, thus removing the stimulus for antibody production. It could perhaps be considered that the organisms are located in areas of relatively good rather than poor blood supply, or are extracellular rather than intracellular. Thus, when the serum agglutinating level can be reduced, it indicates that sufficiently high concentration of the drug can reach the infected area resulting in a reduction in the number of organisms. Indirectly then, the stimulus for agglutinin production will be reduced

due to the presence of fewer organisms. Conversely, when the titer is not affected, it indicates that sufficient drug does not reach the organisms thus permitting the stimulus for antibody production.

This might explain why various investigators obtained different results when working with furazolidone and *S. pullorum*. Gordon and Tucker[1,2] could have been working with birds in a bacteremic or acute form of the disease. The organisms were present in large numbers as evidenced by the high titers involved and were in areas of the body where high concentration of the drug could reach and kill the organisms thus removing the stimulus for antibody production. On the other hand, in the birds used by Wilson,[8] the organisms might have been located in areas of the body where the drug could not destroy the bacteria and thus did not remove the stimulus for antibody production.

Symser and Van Roekel[5] were able to recover *S. pullorum* from birds which had not developed detectable titers. The drug probably kept the number of organisms down to a level where agglutination titers were not produced. In both experiments reported in this paper, standard strain *S. pullorum* was recovered from birds which had no detectable agglutination titers. It should be noted that the antigen used contained standard strains of *S. pullorum*.

In the two experiments reported in this paper, three infected birds became positive serologically with the recovery of *S. pullorum* 3 to 6 weeks after the removal of the drug. Smyser and Van Roekel[6] reported similar results in their experiment with furazolidone at the .011 percent level. It is evident that medication with furazolidone may interfere with the control of pullorum disease in chickens by the agglutination test. In one experiment reported in this article a reactor developed 6 weeks after treatment had been suspended. In this trial birds were not examined over a longer time. Although birds were examined for 10 weeks in the second trial without development of agglutination titer it is impossible to state with certainty how long one must withhold medication before the results of the agglutination test are meaningful.

SUMMARY

A group of 14-week-old Single Comb White Leghorns and a group of 9-week-old Vantress-Arbor Acre cross chickens were infected orally with *Salmonella pullorum*. The birds were fed an all purpose ration with and without .011 percent furazolidone. Tube agglutination titers against *S. pullorum* were conducted weekly and four birds were sacrificed for bacteriological cultures each week.

Salmonella pullorum was recovered from two furazolidone treated birds which had not developed a detectable agglutination titer against pullorum antigen. Two birds developed agglutination titers while the birds were receiving furazolidone. *S. pullorum* was recovered from one of these. Three weeks after removal of furazolidone, two birds which had been negative to the tube agglutination test became positive and *S. pullorum* was recovered from them. Six weeks after removal of the drug, another bird became positive to the agglutination test and *S. pullorum* was recovered.

It was concluded that blood samples for pullorum tests should not be drawn until furazolidone had been removed from the ration for at least six weeks. Since the experiments reported in this article only cover a period of six and ten weeks after medication, the possibility exists that a longer waiting period might be necessary.

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A CARDIAC BLEEDING TECHNIQUE FOR SMALL BIRDS

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SEVERAL methods of bleeding small birds have been published. Sooter[2] used the cardiac puncture. McClure and Cedeno recommended the right jugular vein. The method here described is one that can be effected by one person, is rapid and permits the removal of blood under aseptic conditions.

The wings are placed together and fastened above the back by means of a laboratory hose pinch clamp applied at the primary feather area (figure 1, right). A slot, 1/4 to 3/8 inches x 2 to 3 inches is made in a 4 x 5 inch piece of cardboard or hard board. The wings are slid in this slot with the clamp on the underside. The birds feet may be secured by means of a rubber band or by a loop of cord run through a one holed rubber stopper with the loop placed over the legs and stopper

Fig. 1. Technique for bleeding small birds

pushed up snugly. The bird on the support board is placed on a suitable sized box, bird uppermost. The bird's neck is positioned over the edge of the support. An alcohol soaked cotton swab is used on the suprasternal cleft. Puncture is readily effected (figure 1, left) by one person. One finger of the hand holding the syringe barrel can be used to keep the head down while the other hand is free to manipulate the syringe plunger.

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Fig. 1. Restraint of small birds for cardiac bleeding.

THE *IN VITRO* NEUTRALIZATION TEST WITH AVIAN ENCEPHALOMYELITIS VIRUS (AEV)‡

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THE *in vivo* virus neutralization test has been accomplished in chicks[4] and chicken embryos[1,5] to assay serum potency against avian encephalomyelitis (AEV). The cytopathogenicity of AEV in chick kidney cell culture has also been reported.[3] This communication reports the utilization of the serum neutralizing capacity against the cytopathogenicity of the virus *in vitro* as a means of serum potency assay.

MATERIALS AND METHODS

Pooled serum samples were collected from 30 field cases of avian encephalomyelitis* (AE). Serum of five hens and 36 eggs were taken from each of 12 breeding flocks and six groups of normal chickens of known history aged 2, 4, 6, 8, and 14 weeks were also bled for serum. Seven samples of anti-AEV hyperimmune serum were also included. All serum samples were stored at –20 C until tested. The eggs were incubated for the embryo susceptibility test.[6] Virus neutralization tests[3] were performed in chick kidney cell culture in tubes[2] using stock virus of the chick kidney cell culture 10th passage, the titer of which was 10^8 cell culture infective $dose_{50}$ ($CCID_{50}$) per 0.1 ml.

‡ Supported in part by funds from the American Poultry and Hatchery Federation.

* Diagnosis made by the Department of Animal Diseases, University of Connecticut.

RESULTS

The results of virus neutralization tests with serum samples from field cases, normal, and hyperimmune chickens are summarized in table 1; the results with the serum samples from breeding flocks and parallel embryo susceptibility tests are presented in table 2. Of the 30 serum samples from field cases, 19 or 63.3 percent neutralized $10^{2.0}$ to at least $10^{4.5}$ $CCID_{50}$ and 11 or 36.7 percent neutralized at most $10^{1.5}$ $CCID_{50}$ of the stock virus. Six serum samples from normal chickens neutralized $10^{1.0}$ to $10^{1.6}$ $CCID_{50}$ and seven samples of homologous hyperimmune serum neutralized $10^{6.5}$ to $10^{8.5}$ $CCID_{50}$ of the stock virus. Eleven serum samples from breeding flocks, the embryos of which yielded virus titers* of $10^{-1.5}$ to $10^{-3.0}$, neutralized 10^{4} to 10^{5} $CCID_{50}$. The virus titer in embryonated eggs from one flock was $10^{-5.4}$ and the serum samples from this flock neutralized 10^{2} $CCID_{50}$.

TABLE I

Results of AEV neutralization tests with field cases, normal, and hyperimmune serums

Serum	Number of samples	Virus neutralizing doses
Field case	11	at most $10^{1.5}$
	2	$10^{2.0}$
	3	$10^{2.5}$
	1	$10^{3.0}$
	1	$10^{3.5}$
	1	at least $10^{4.0}$
	1	at least $10^{4.3}$
	10	at least $10^{4.5}$
Normal	3	$10^{1.0}$
	2	$10^{1.3}$
	1	$10^{1.6}$
Hyperimmune	2	$10^{6.5}$
	4	$10^{7.5}$
	1	$10^{8.5}$

DISCUSSION

Six serum samples from normal chickens, 2 to 14 weeks old, and 11 serum samples from field cases of 2 to 3-week-old chicks neutralized $10^{1.0}$ to $10^{1.6}$ $CCID_{50}$ of the stock virus indicating that serum of young chicks contains very small

* Reported by Mr. F. W. Sumner, Virus Laboratory, Department of Animal Diseases, University of Connecticut.

TABLE II

Results of virus neutralization tests with serum samples from breeding flocks and parallel embryo susceptibility tests

Flock number	Virus neutralizing doses	Virus titer in embryonated eggs*
1	at least 10^5	$10^{-1.7}$
2	at least 10^5	$10^{-3.0}$
3	at least 10^5	$10^{-1.9}$
4	at least 10^4	$10^{-2.7}$
5	at least 10^5	$10^{-2.4}$
6	at least 10^5	$10^{-2.6}$
7	at least 10^5	$10^{-2.8}$
8	10^2	$10^{-5.4}$
9	at least 10^5	$10^{-1.5}$
10	at least 10^5	$10^{-1.8}$
11	at least 10^4	$10^{-1.9}$
12	at least 10^5	$10^{-1.6}$

* Reported by Mr. F. W. Sumner, Virus Laboratory, Department of Animal Diseases, University of Connecticut.

amounts of anti-AEV substances. Chick serum, which neutralizes 10^2 $CCID_{50}$ or more of the virus, suggests the possibility of a previous infection, a parallel seen in human poliovirus infection.[7] If the assumption is valid that a virus titer of $10^{-3.5}$ or lower in embryonated eggs is an indication of resistance against AEV of immune nature,[6] then the results of virus neutralization tests with serum samples from breeding flocks are in full agreement with those of the parallel embryo susceptibility tests. Thus the *in vitro* virus neutralization test may be employed as an assay technique for evaluating the serum potency against AEV.

SUMMARY

In vitro virus neutralization tests against avian encephalomyelitis virus were performed with pooled serum samples from 30 field cases of avian encephalomyelitis, 12 breeding flocks, and six groups of normal chickens together with seven homologous, hyperimmune, serum samples. The results indicated that the technique may be useful for the assay of serum potency against AEV.

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TISSUE CULTURE AND CHICKEN EMBRYO TECHNIQUES FOR INFECTIOUS LARYNGOTRACHEITIS VIRUS STUDIES*

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INFECTIOUS laryngotracheitis (ILT) was first reported by May and Tittsler.[11] The viral etiology of the disease was later established by Beach[2] and the propagation of the virus on chicken embryo chorioallantoic membrane (CAM) was de-

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scribed by Brandly.[3] Burnet,[4] Pulsford and Stokes,[13] and more recently Hitchner *et al.*[10] used the CAM technique to perform serum neutralization tests. The propagation of ILT virus in cell culture of chicken-embryo-lung and the neutralization of the virus by immune serum has previously been reported by Pulsford[12] and Atherton and Anderson.[1] Atherton and Anderson[1] also reported that the virus caused plaques in cell cultures of chicken embryo tissue. This communication reports the successful propagation of ILT virus in chicken-embryo-kidney cell culture and the demonstration of virus neutralization (VN) using the cell culture plaque technique.[7] The VN test in tissue culture was compared with a CAM technique similar to that of Hitchner and co-workers[10] and a modification of the chorioallantoic sac (CAS) technique described by Fabricant[8] for infectious bronchitis.

MATERIALS ND METHODS

Virus. Six ILT viruses were tested for propagation in cell cultures. Three were obtained from vials of commercial vaccines, two were isolated from field cases in Connecticut, and a mild strain, isolated from a field case in Delaware was received from Dr. M. S. Cover, University of Delaware, Newark, Delaware.

Cell culture media. The wash medium contained Hanks' basic salt solution (BSS)[9] buffered with 0.5 percent of 7.5 percent sodium bicarbonate and 0.5 percent lactalbumin hydrolysate (LAH) adjusted to pH 7.3 with 4 percent sodium hydroxide. The LAH was prepared as a 5 percent solution and sterilized at 10 pounds steam for 10 minutes. Two hundred units of penicillin, 200 μgm of streptomycin, and 100 units of mycostatin were added per ml of medium.

Growth medium contained 95 percent wash medium and 5 percent calf serum. The maintenance medium was the same as the wash medium except that the percentage of sodium bicarbonate was doubled.

The overlay medium contained 2.4 percent of 1:1000 neutral red, 0.5 percent LAH, 1 percent of 7.5 percent sodium bicarbonate, 5 percent calf serum, and 1.5 percent purified

agar in Hanks' BSS without phenol red. Antibiotics were added in the same concentration as above.

In preparing 300 ml of the overlay medium, 4.5 gm agar was mixed with 100 ml water and autoclaved at 12 pounds steam for 15 minutes and then placed in a water bath at 43 C for 1 hour. The remaining ingredients were mixed as follows and maintained at 37 C. Two hundred ml of solution was made by combining 30 ml of 10 X concentrated Hanks' BSS without phenol red, 7.2 ml of 1:1000 neutral red, 30 ml of 5 percent LAH, 3.0 ml of 7.5 percent sodium bicarbonate, 15 ml of calf serum, and 109.8 ml of distilled demineralized water. All the above mentioned ingredients were sterilized prior to mixing. At the end of the 1 hour water bath immersion, the two mixtures were combined, and the medium was maintained at 38 C until dispensed over the cell sheets. After remaining at room temperature for 45 minutes, the bottles were inverted and incubated at 37 C.

Cell cultures. Kidneys were removed aseptically from exsanguinated 20-day-old chicken embryos, thoroughly minced with surgical scissors, and then washed in Dulbecco's phosphate-buffered saline (PBS)[6] to remove excessive blood and debris. A suspension was made using 8.0 ml of a solution of 0.25 percent trypsin in PBS at 4 C for each pair of prepared kidneys. The suspension was kept stoppered at 4 C for approximately 16 hours (overnight). It was then shaken vigorously and filtered through eight layers of 20/12 sterile gauze, centrifuged at 1000 rpm for 10 minutes, and decanted. The cells were then resuspended in the wash medium. After a second centrifugation, the cells were resuspended in growth medium at 20 ml per pair of kidneys. The suspension was then filtered through 16 layers of 20/12 sterile gauze and seeded at 8.0 ml per 3 ounce prescription bottle. The bottles were sealed with white rubber stoppers and incubated at 37 C. Cell sheets formed in 3 to 5 days (figure 1), at which time the medium was replaced with maintenance medium or agar overlay medium.

Serum. A pool of normal serum was obtained from 12-

week-old White Rock (WR) birds prior to inoculation. The birds were then inoculated with undiluted ILT virus directly into the infraorbital sinus or cloaca and bled for a pool of ILT positive serum 4 weeks after initial inoculation. Positive serum was prepared for two strains to be used for comparison.

Virus propagation and neutralization on CAM. The viruses were propagated on the CAM and the VN tests were conducted as described by Hitchner *et al.*[10] However, in this work, the CAM tissue was ground with a Ten Broek tissue grinder and the serum was not heat-inactivated for the neutralization test. CAM material was used to infect cell cultures and birds for immunization.

Virus propagation and neutralization in cell culture. One ml of CAM material was placed as inoculum in 9.0 ml of maintenance medium which was added to a bottle containing a sheet of chicken-embryo-kidney cells decanted of medium. When 50 to 75 percent of the sheet became infected, the bottle was shaken vigorously and the cells were flushed off the inside surface and broken up thoroughly with a 10 ml pipette before being aspirated. This suspension was used to repass the virus in cell cultures and to inoculate into the CAS of chicken embryos.

Each strain of virus was passed at least four times in cell cultures as a 10^{-1} dilution. A virus grown from a commercial vaccine (C_1) was selected for the serologic study because it grew more rapidly than the others.

This ILT (C_1) virus produced plaques in cell culture. Therefore, a VN test was attempted by this method. Tenfold serial dilutions of the 10th cell culture passage virus were prepared. Equal volumes of virus dilution and serum were thoroughly mixed and both preparations were incubated at room temperature for 1 hour. The cell sheets were then inoculated with either 0.1 ml of virus dilution or 0.2 ml of serum-virus dilution mixture and incubated for 1 hour at 37 C. The overlay medium was then added.

Virus propagation and neutralization in CAS. The first cell culture passage of this virus was lethal for chicken em-

bryos via CAS inoculation and was carried through eight passages as allantoic fluid (AF) diluted 10^{-2} in tryptose broth. Infected AF was then used in a neutralization test as described by Fabricant[8] for infectious bronchitis except that the serum virus mixture and the virus titration, mixed in tryptose broth, were incubated at room temperature for 1 hour before inoculating embryos.

Virus storage. In most instances the harvested virus material was mixed 11 with 60 percent glycerin. This mixture was placed in air-tight, screw cap vials and frozen at –40 C. The glycerin was prepared in distilled water, buffered with KH_2PO_4, and adjusted to pH 7.5 with KOH.

Infection of birds and CAM with cell culture virus. The CAM of 9-day-old chicken embryos was inoculated with undiluted 10th cell culture passage virus. Portions of infected CAM were fixed in acidified Zenker's fixative daily, from the 1st to the 5th day postinoculation. Twenty-week-old WR birds were inoculated intrasinusoidally with the same undiluted virus and portions of beak were placed in acidified Zenker's fixative daily, from the 1st to the 9th day and every 3 days thereafter to 21 days postinoculation. All fixed tissues were subjected to routine dehydration and eventual embedding in paraffin, cut at six microns, and stained by hematoxylin and eosin.

Permanent slides. Permanent slides of cell cultures, prepared as described in a previous publication,[5] showing the cytopathogenic effect (CPE) of ILT virus were made.

RESULTS

All six strains of ILT virus produced CPE (figure 2) on initial passage in chicken-embryo-kidney cell cultures 48 to 96 hours after inoculation with the CAM material. The CPE produced by ILT virus is similar to that caused by avian infectious bronchitis virus.[5] The virus caused groups of cells to undergo even, granular degeneration while the periphery of this mass became sharply limited. Eventually the cells either fell from the glass surface or thinned out into a spiderweb-like structure.

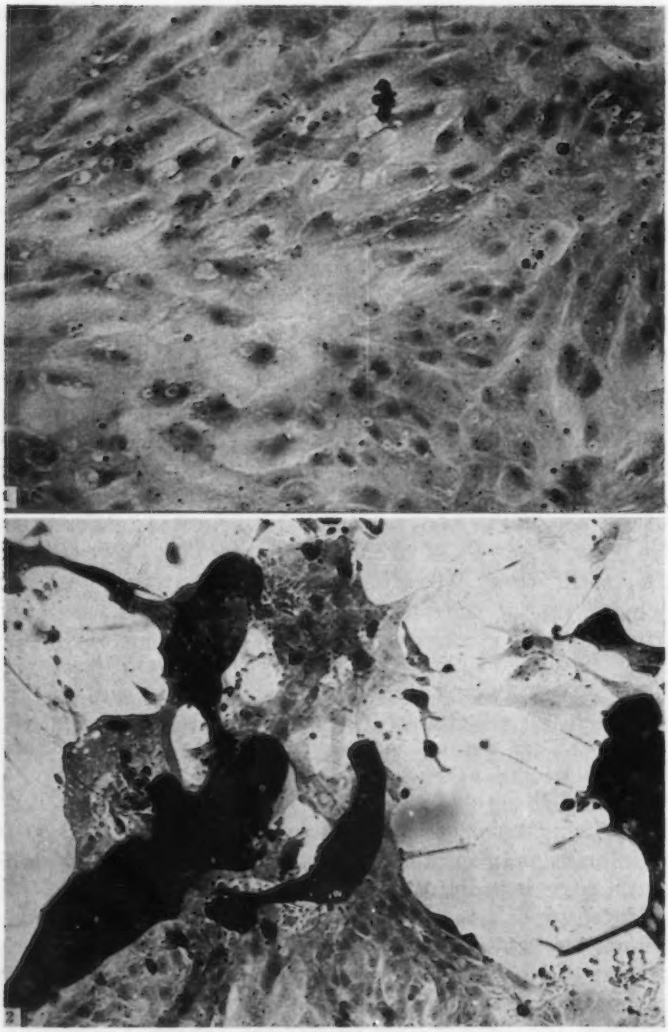

Fig. 1. (top) Normal chicken-embryo-kidney cell culture. Hematoxylin and eosin stain. X200.

Fig. 2. (bottom) Chicken-embryo-kidney cell culture 36 hours after inoculating with ILT. Most cells have coagulated to form a granular mass. Hematoxylin and eosin stain. X200

On subsequent passage at 10^{-1} the initiation of CPE for all six strains varied from 36 to 48 hours postinoculation, and the time for completion varied from 72 to 120 hours.

The 10th cell culture passage of C_1 virus formed plaques on chicken-embryo-kidney cells and contained 10^5 plaque forming units (PFU) per ml. Plaques could be counted 5 days postinoculation, at which time they were 1 to 2 mm in diameter. By the 10th day, all plaques had appeared and attained a diameter of 4 to 5 mm. Thirty days postinoculation, the plaques had reached a maximum diameter of 10 mm. Plaques produced by ILT virus had an irregular edge as shown in figure 3.

Fig. 3. Titration of ILT virus in chicken-embryo-kidney cell culture by the plaque method.

In tissue culture titration of ILT (table 1), the undiluted virus destroyed the cell sheet before plaques could be formed; 10^{-1} virus dilution formed too many plaques to be counted as they appeared confluent; higher dilutions formed countable plaques which diminished approximately in 10-fold numbers as the comparable dilutions increased. ILT antiserum neutralized up to $10^{4.1}$ cell culture PFU.

TABLE I

Virus neutralization titers by tissue culture plaque count

		Dilutions							
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	PFU/ml	VND
Trial 1	C_1 virus	—	TNC*	120	12	1	0	$10^{5.1}$	
	Normal serum	—	TNC	110	19	0	0	$10^{5.1}$	10^{0}
	C_1 antiserum†	40	4	0	0	0	0	$10^{2.6}$	$10^{2.5}$
	C_2 antiserum††	27	2	0	0	0	0	$10^{2.4}$	$10^{2.7}$
Trial 2	C_1 virus	—	TNC	118	20	0	0	$10^{5.1}$	
			TNC	115	20	1	0		
			TNC	148	14	0	0		
	Normal serum	—	TNC	108	9	0	—	$10^{5.0}$	$10^{0.1}$
			TNC	100	22	1	—		
			TNC	117	18	0	—		
	C_1 antiserum†	117	5	0	0	—	—	$10^{3.0}$	$10^{2.1}$
		103	5	0	0	—	—		
		111	7	0	0	—	—		
Trial 3	C_1 virus	—	TNC	125	16	2	0	$10^{5.1}$	
	Normal serum	—	TNC	200	30	7	0	$10^{5.4}$	10^{0}
	C_1 antiserum††	1	0	0	0	—	—	10^{1}	$10^{4.1}$

*TNC = Too numerous to count.
† Birds immunized with cell culture virus.
†† Birds immunized with CAM passed virus.
0 = no plaques formed.
— = not done.

The first cell culture passage of C_1 virus was lethal for 9-day-old chicken embryos via CAS inoculation, and the eighth passage AF contained $10^{6.2}$ ELD_{50} per ml. When infected embryos were examined they appeared dwarfed and the CAM contained numerous plaques. Embryos inoculated with virus dilutions higher than the terminal infective dilution had no apparent lesions, attained normal size at 21 days, and the majority were able to pip or hatch.

In serial passage of the virus, AF was harvested after death of the embryo up to 7 days postinoculation (table 2). After the eighth passage, infective AF was harvested from living embryos 3 days postinoculation. The titer of the 10th passage in the CAS of chicken embryos was $10^{-6.7}$ per ml. Since the virus required at least 4 days to begin killing embryos they were inoculated at 9 days of age and kept until 10 to 20 days old to complete the VN test. ILT antiserum neutralized up to $10^{4.5}$ ELD_{50}.

The 10th cell culture passage virus produced acidophilic

TABLE II

Death pattern of chicken embryos inoculated into the chorioallantoic sac with laryngotracheitis virus

Passage	Inoculum	Dilution inoculated	Number of embryos inoculated	Number of embryos dead days postinoculation						
				1	2	3	4	5	6	7
	Cell	10^{-0}	3	0	0	0	1	2	0	0
	culture	10^{-1}	3	0	1	0	0	1	0	1
1st	fluid	10^{-2}	3	0	0	0	0	0	1	2
		10^{-1}	3	0	0	0	2	0	1	0
2nd	AF	10^{-2}	3	0	0	0	1	0	2	0
		10^{-3}	3	0	0	0	1	0	1	1
		10^{-1}	3	0	0	0	3	0	0	0
3rd	AF	10^{-2}	3	0	0	0	1	1	1	0
		10^{-3}	3	0	0	0	0	0	3	0
		10^{-1}	3	0	0	0	0	3	0	0
4th	AF	10^{-2}	3	0	0	0	0	2	1	0
		10^{-3}	3	0	0	0	0	0	2	1
		10^{-1}	3	0	0	0	2	1	0	0
5th	AF	10^{-2}	3	0	0	0	1	2	0	0
		10^{-3}	4	0	0	0	3	0	0	0
6th	AF	10^{-2}	4	0	0	1	0	3	0	0
7th	AF	10^{-2}	4	0	0	0	0	2	2	0
8th	AF	10^{-2}	4	0	0	0	0	2	2	0

intranuclear, inclusion bodies in the CAM. Questionable inclusion bodies were seen 2 days postinoculation in the sinus tissue of 20-week-old birds which had been inoculated, via the infraorbital sinus, with 10th cell culture passed virus. In this group of birds, swelling of the infected sinus appeared 2 days postinoculation and lasted 5 days. Serum from these birds showed a significant rise in VN titer 4 weeks after inoculation: $10^{2.5}$ VND_{50} by the cell culture plaque method, $10^{3.4}$ VND_{50} by the CAS method, and $10^{3.3}$ VND_{50} by the CAM method (table 3).

Another serum, prepared in birds against C_1 virus (CAM propagated) by vent drop vaccination,[10] followed at 3 weeks by sinus challenge, contained $10^{4.1}$ VND_{50} by the cell culture plaque method, 10^{4} VND_{50} by the CAS method, and $10^{3.7}$ VND_{50} by the CAM method (table 3). Serum prepared against another commercial virus C_2 (CAM propagated), by sinus inoculation into birds, contained $10^{2.7}$ VND_{50} by the cell culture plaque method, $10^{4.5}$ VND_{50} by the CAS method, and 10^{4} by the CAM method (table 3).

TABLE III

Comparative neutralization indexes of sera tested in chicken embryos and cell cultures

Antiserum	C_1 virus		
	In cell cultures	In embryos (CAS)	In embryos (CAM)
C_1†	$10^{2.5}$*	$10^{3.4}$	$10^{3.3}$
C_1†	$10^{2.1}$**	—	—
C_1††	10^{4-1}*	10^{4}	$10^{3.7}$
C_2††	$10^{2.7}$*	$10^{4.5}$	10^{4}

† Birds immunized with cell culture virus.
†† Birds immunized with CAM passed virus.
* One bottle used per dilution.
** Three bottles used per dilution.
— Not done.

DISCUSSION

ILT virus appeared to inhibit the metabolism of chicken-embryo-kidney cells. Affected cultures showed little color change, indicating no pH change, while control cultures showed a rapid change. Control cultures were kept 7 to 9 days before degeneration and peeling of the sheet occurred.

Since every strain of ILT virus used in this study propagated and caused CPE in cell cultures, it is conceivable that many strains may have the same property. If this is true then cell cultures might also be used for primary isolation of the virus from tracheal material taken from infected birds.

Plaque formation by ILT virus makes available a method for obtaining pure strains by establishing a pure clone of virus propagated from one discrete isolated plaque.

Since plaque formation by ILT virus in cell culture was stopped by specific antiserum and not by normal serum, a VN test was indicated. Specific ILT antiserum also neutralized the lethal effect of the CAS passed virus as well as the pock forming ability of the CAM passed virus. Therefore, three serologic methods are available for testing the ILT virus antibody level of birds. The CAS test is the easiest to perform while the tissue culture plaque test appears to be the least sensitive.

The C_1 virus previously propagated on CAM produced embryo mortality when inoculated via the CAS after one passage in cell culture. Further work is in progress to attempt similar egg adaptation of other isolates.

ACKNOWLEDGMENT

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SUMMARY

Three commercial strains and three field strains of infectious laryngotracheitis (ILT) virus caused a cytopathogenic effect in chicken-embryo kidney cells on first passage. The virus caused groups of cells to undergo even, granular degeneration and the periphery of this mass to became sharply limited.

The 10th cell culture passage of a commercial strain had 10^5 plaque forming units per ml when titered in tissue culture. This passage caused intranuclear inclusion bodies (INIB) in chorioallantoic membrane (CAM) and proved infective for birds when inoculated into the infraorbital sinus. Birds inoculated intrasinusoidally showed swelling of the sinus and a rise in antibody titer.

First cell culture passage of this commercial strain of ILT virus killed embryos inoculated into the chorioallantoic sac (CAS). The eighth passage CAS material contained $10^{6.2}$ ELD_{50} per ml.

Virus neutralization tests were performed using the cell culture plaque method, the chicken embryo allantoic sac, and the chorioallantoic membrane method. The CAS method was as sensitive as the CAM method but the cell culture plaque method appeared less sensitive than either of these.

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EASTERN ENCEPHALITIS IN WHITE PEKIN DUCKLINGS ON LONG ISLAND

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EQUINE encephalomyelitis was first described in the United States in the fall of 1912 by Udall.[45] The eastern type was differentiated by Ten Broeck and Merrill in 1933. Sporadic outbreaks of the eastern type have occurred east of the Appalachian mountains since that time. Many of these outbreaks have been reported in the states bordering New York (Connecticut,[30,44] Pennsylvania[6] and New Jersey[3,4,5,6,7,8]). However, it was not until October 1952 that a case was reported in New York state, when Dr. C. I. Angstrom of the Kingston Regional Poultry Laboratory reported the disease in Orange county pheasants.[2] Virus isolation from these pheasants was reported by Beaudette[7] in 1954. Since that time, no further cases have been reported in New York state in pheasants or any other species of animal. Clinical human cases of the disease have never been recorded in New York.

Although eastern equine encephalomyelitis was first described in horses,[45] the wide host range renders the term equine inappropriate and the name eastern encephalitis (EE) has been adopted by virologists and will be used in this paper. The extent of the host range is indicated by the fact that the following species have been experimentally or naturally in-

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fected with the eastern encephalitis virus: horse,[32] mule,[34] mouse,[40] rat,[32] rabbit,[40] man,[34] guinea pig,[40] calf,[43] sheep,[43] ground squirrel,[34] puppy,[34] cat,[34] pig,[34] hedgehog,[34] gopher,[34] hamster,[34] monkey,[34] deer,[34] goat,[34] redwing blackbird[14] cardinal,[27] English sparrow,[46] American egret,[27] snowy egret[27] white ibis,[27] mourning dove,[14] purple grackle,[26] cowbird,[14] pigeon,[17] yellow crown night heron,[38] mocking bird,[37] green heron,[37] Louisiana heron[37] little blue heron,[38] white-throated sparrow,[38] blue-jay,[38] tufted titmouse,[38] meadow lark,[38] cat bird,[37] lesser yellowleg,[37] Carolina chickadee,[37] song sparrow,[38] towhee,[38] hermit thrush,[38] brown thrasher,[38] stork,[33] eastern kingbird,[37] yellow-throated vireo,[37] Carolina wren,[37] guinea fowl,[36] chicken,[42] turkey,[47] pheasant,[44] northern waterthrush,[37] white-eyed vireo,[37] Kentucky warbler,[37] quail,[34] ruby-crowned kinglet,[38] Myrtle warbler,[38] rufouse-sided towhee[38] brown-headed cowbird,[38] screech owl,[37] and black-crowned night heron.[26]

There is evidence that EE may spread in pheasants by ingestion of contaminated material such as feathers[8] and by direct contact under laboratory conditions.[20] However, the evidence continues to be overwhelmingly in favor of insect spread. Horses were found to circulate virus in the blood[18,41] and Kelser[24,25] in 1933 demonstrated that mosquitoes may serve as vectors. Such biting insects as horseflies, stable flies, and deer flies have been reported as mechanical carriers by Chamberlain.[13] *Culicoides,*[23] and the following species of mosquitoes have been incriminated as carriers: *Aedes aegypti,*[11] *Aedes atropalpus,*[11] *Aedes cantator,*[31] *Aedes mitchellae,*[23] *Aedes solicitans,*[11] *Aedes triseriatus,*[11] *Aedes vexans,*[11] *Anopheles crucians,*[27] *Culex erraticus,*[11] *Culex quinquefasciatus,*[35] *Culex restuans,*[11] *Culex salinarius,*[11] *Mansonia perturbans,*[21] *Orthopodomyia signifera,*[11] *Psorophora ciliata,*[11] *Psorophora confinnis,*[11] *Psorophora ferox.*[11] However, it seems probable that in ducks, the transmission must be attributed to *Culiseta melanura,* the fresh water swamp mosquito, which is considered the primary sylvan vector. Frequent virus isolations have been made from adult *Culiseta melanura*[1,10,19] and H. Jamnback[22]

has pointed out the wide distribution and large numbers of *Culiseta melanura* in Suffolk County, Long Island.

CASE REPORT

On July 18, 1959, a new disease syndrome was observed in a flock of 2-week-old White Pekin ducklings reared on Long Island (accession #5409). The disease was characterized by a bilateral, posterior paresis followed by complete paralysis and the absence of gross lesions at necropsy. Thirteen ducklings died in a flock of 2200 over a 3 day period. Attempts to reproduce the condition by intramuscular and intratracheal inoculation of ground spleen into 3-week-old ducklings were unsuccessful. Changing the feed did not appear to have any effect and consumption of feed from this farm by research farm ducklings had no effect. Testing of the feed was considered necessary in view of the finding of a feed induced, reproducible, toxic condition in several flocks of ducklings during the 1958 growing season. This toxemia was called "creeper disease" by the duck farmers and will be more completely described under differential diagnosis. During the latter part of July and early August, birds exhibiting similar signs were submitted to the laboratory by two other farms. Feed tests, animal inoculations, and virus isolation attempts were negative despite the fact that in one flock losses were 50 percent.

On August 17, 1959, ducklings with similar signs were received from a fourth farm. A filterable agent which was lethal for chick and duck embryos in 15 to 48 hours and weanling white mice in 4 days, was recovered from these ducklings. This agent was subsequently identified as eastern encephalitis virus and confirmed by Dr. D. D. Stamm of the Virus and Rickettsia Section of the Communicable Disease Center in Montgomery, Alabama.

Through September and October outbreaks of a similar disease occurred on six additional, widely scattered Long Island duck farms and the EE virus was recovered from four of these. In all, 18 flocks on ten farms were involved and virus isolations were made from eleven of the 18 flocks on five of the ten farms.

Signs. A bilateral, posterior paresis followed by paralysis was the most consistent sign. The natural disease was not seen in ducklings older than 18 days of age and laboratory attempts to infect older ducklings with overwhelming doses, up to a billion embryo lethal doses, gave erratic results. Chamberlain[12] found that the half-day-old (wet) chicks were more uniformly susceptible to the eastern encephalitis virus than day-old-chicks. In ducklings, the susceptibility was inversely proportional to the age.

The affected ducklings remained bright-eyed up to the time of death. A few developed opisthotonus similar to that described in virus hepatitis[29] of ducks. Morbidity was high (over 50 percent) in only five of the eighteen flocks. Mortality varied between 2 and 60 percent. Very few of the affected birds recovered but, some of the mortality may have been the result of water starvation. Ducklings in pens separated from affected birds of the same age by a wire fence, frequently remained clinically free of the disease.

Gross lesions. Typical of eastern encephalitis infection, characteristic gross lesions were lacking. Eighteen of 262 suspect ducklings examined had hematomas beneath Glisson's capsule. Many had a secondary nephrosis as the result of water starvation.

Microscopic lesions. The most consistent lesion was an edema of the white matter of the spinal cord (figure 1), especially in the ventral columns. Occasionally, a lymphocytic cerebellar or cerebral leptomeningitis (figures 2, 3, and 4) was encountered. A mild gliosis was usually present. A lymphocytic perihepatitis was observed in one bird from a field case complicated by *Pasteurella anatipestifer* infection.

Diagnosis. The characteristic paralysis in the absence of gross lesions should lead one to suspect this disease in ducks. Confirmation would have to be made by virus isolation and serum neutralization in weanling mice, chicken or duck embryos, or tissue culture. Recovered ducklings develop sufficiently high SN titers (4 to 5 logs) to establish a diagnosis.

Duck embryos inoculated with the virus had a hyperemia as seen in chick embryos and also developed a hyperplasia of

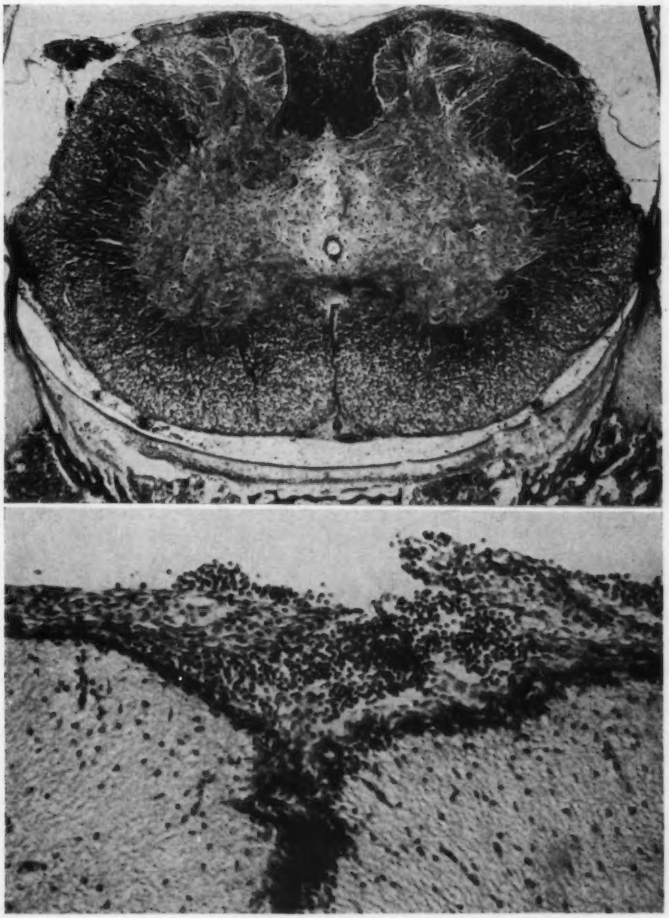

Fig. 1. (top) Histologic cross section in the lumbar region of the spinal cord of a moribund eight-day-old duckling from a natural case of eastern encephalitis. Note the extensive edema of the white matter, particularly the ventral columns. Hematoxylin and phloxine (X50).

Fig. 2. (bottom) Histologic section of the cerebellum of a moribund 7-day-old duckling experimentally infected with eastern encephalitis. Note the leptomeningitis with lymphocytic inflammatory cells predominating. Hematoxylin and phloxine (X220).

the chorioallantoic membrane at the site of inoculation. Carneiro[9] produced a similar lesion when he inoculated duck embryos with the Brazilian strain of the encephalitis virus.

Differential diagnosis. Several complicating diseases were found in the flocks which had EE, notably, *Pasteurella anatipestifer* infection (infectious serositis), virus hepatitis, and paratyphoid. However, all three of these diseases produce characteristic gross lesions,[15,16,29] which makes differential diagnosis relatively easy.

The use of .0025 percent of 3-nitro-4-hydroxyphenylarsonic acid in duck feeds produces a posterior paralysis. This paralysis, in contrast to EE, does not develop until the birds are about 4 weeks of age and was noticeable only when the birds were driven.

The most confusing diagnostic picture was presented by the so-called "creeper disease" which was mentioned previously. During the 1958 growing season 8-day-old ducklings on at least four farms developed a lameness which was similar to that described for EE. The cause was found to be a toxic factor in the feed. Recovery was almost immediate when the feed was removed. Recovery from EE paralysis is slower. We were able to reproduce "creeper disease" in laboratory ducklings by feeding samples of the toxic feed. Analysis of the feed for arsenic, excessive salt and strychnine was negative and to date the exact nature of the toxic substance remains unknown.

Diagnosis in species other than ducks. During the 4 months covered by the duck outbreak, virus isolation attempts were made from 23 pheasants received at the laboratory for routine necropsy examination from three Long Island farms. EE virus was recovered from one bird. Several birds in the positive flock exhibited the classical signs of EE. The virus was isolated on September 24, 1959 and confirmed at the Communicable Disease Center. This outbreak involved 200 pheasants (two pens) in a flock of 3200 (thirteen pens). Seventy birds died during the outbreak.

Unsuccessful virus isolation attempts were made from sparrows, sandpipers, and a long eared owl collected on Long Island.

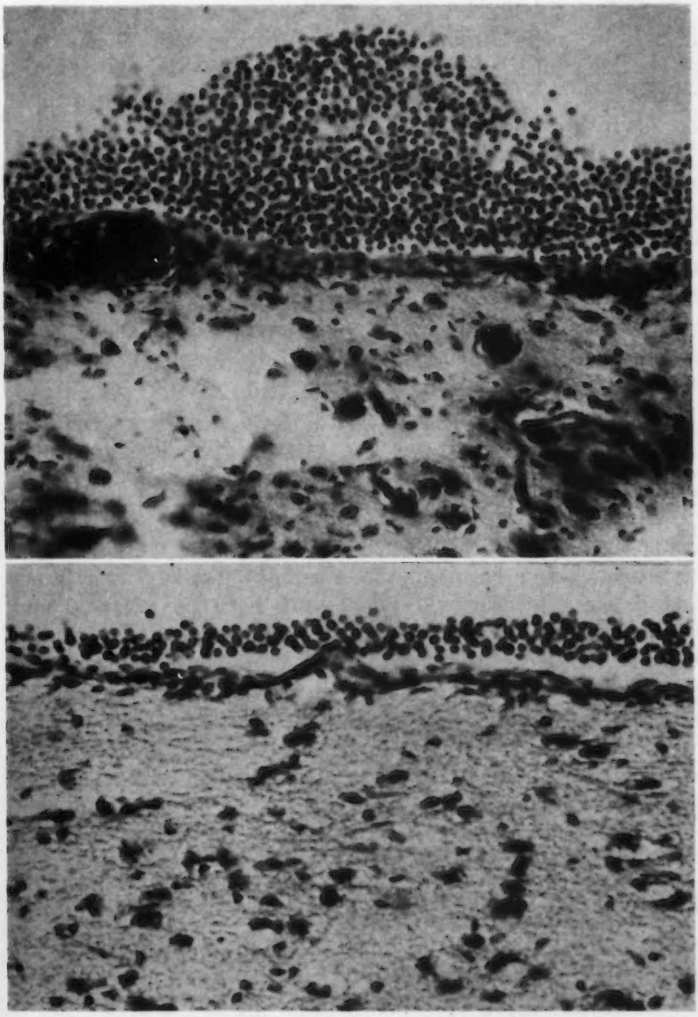

Fig. 3. (top) Histologic section of the cerebrum of a moribund 3-day-old duckling experimentally infected with eastern encephalitis. Note the extensive, lymphocytic leptomeningitis. Hematoxylin and phloxine (X220).

Fig. 4. (bottom) Histologic section of a cerebrum of an 8-day-old duckling from a field case of eastern encephalitis. The lymphocytic leptomeningitis is less extensive than in the experimental infection (fig. 3). Hematoxylin and phloxine (X500).

Prevention and control. Preventive measures are limited at present to the elimination of the insect vectors. Vaccination as recommended in horses[42] and in pheasants[39] may be of some value; however, the young age of the affected ducklings could be a limiting factor.

Treatment of laboratory or naturally infected ducklings with immune sera from recovered ducks did not appreciably alter the mortality. (This practice has been found to be effective in the control of duck virus hepatitis). Losses in the treated ducklings was approximately 7 percent while in untreated ducklings it was about 10 percent in a field case involving 2000 ducklings. In the laboratory, losses were lowered from 100 to 80 percent when day-old ducklings were given a simultaneous serum virus challenge.

EXPERIMENTAL DATA

The ducklings used in our transmission experiments were secured from the experimental farm. The breeder ducks supplying the hatching eggs had no demonstrable serum neutralizing titer against the EE virus. In addition, we decided to secure ducklings from another source 450 miles from Long Island. An infection experiment with both Long Island and upstate ducklings was done and the results are shown in table 1. The upstate ducklings were found to be more susceptible to the EE virus by the intraperitoneal, intramuscular, intranasal, and per os routes. Two of ten uninoculated contact controls in the upstate group died and EE virus was recovered. These birds were housed in a Rockefeller type isolation building which was free of mosquitoes and flies.

TABLE I

Effect of route of inoculation and source of ducklings on mortality from eastern encephalitis

Pen no.	Source of day-old-ducklings	Route of inoculation—2 x 10^5 Embryo LD				
		Intra-perit	Intra-muscular	Intra-nasal	Per os	Contact control
1	Long Island	3/5*	4/5	0/5	0/5	0/10
2	Upper N. Y.	5/5	5/5	5/5	5/5	2/10

* Numerator = Mortality.
Denominator = Number of ducks inoculated.

In cooperation with Dr. Hugo Jamnback of the New York State Museum and C. T. Williamson of the Suffolk County Mosquito Control Commission, we have attempted to isolate EE virus from 1378 *C. melanura* larvae (the overwintering stage) collected in the vicinity of affected duck farms. No virus was isolated.

SUMMARY

The first case of eastern encephalitis in White Pekin ducklings is recorded. The diagnosis was established by virus isolations from ducks on five Long Island farms. The disease was characterized by a sudden onset in ducklings one to 2 weeks of age. A posterior paresis followed by paralysis was the most consistent sign of the disease. Morbidity was usually low (under 50 percent) and mortality varied from 2 to 60 percent. Gross lesions were lacking and microscopic lesions were confined to the central nervous system.

Evidence of contact spread of EE to highly susceptible day-old ducklings is presented. Attempts to isolate the EE virus from *C. melanura* larvae collected in the vicinity of the affected farms were unsuccessful.

An outbreak of eastern encephalitis in farm reared, Long Island pheasants is also recorded. This occurred during the same time that the duck outbreaks were in progress.

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MORTALITY IN WHITE LEGHORN FEMALES ON A FARM IN SOUTHERN ALBERTA

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AUTOPSY records from diagnostic laboratories are often a poor index of the diseases prevalent in the surrounding area and of the economic importance of these diseases. Such information can only be obtained from complete surveys of the causes of mortality in a number of flocks. Detailed examinations of all birds dying on a farm in this area have been

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made since April 1956 and the data may be of interest as there have been few Canadian reports of this kind. Since the results refer to only one farm, they cannot be taken as representative of mortality among chickens in southern Alberta.

An interesting feature about this survey is that as part of another project, half of the flock is maintained on a restricted diet for part of the growing period while the birds are on range. During this time the remaining birds are fed *ad lib*. Up to 30 percent reduction is made in the ration of the restricted group compared with that of the *ad lib*. group. Some interesting differences in the mortality between the two groups have been obtained. The flock under study is composed of many blood lines equally distributed between the two groups. The small numbers of birds in each blood line did not favor an analysis of mortality at this level.

Flock details. Mortality in sexed, late April hatched, White Leghorn pullets, brought in from Ontario in 1956-58 and maintained on the Dominion Experimental Farm, Canada Department of Agriculture, Lethbridge, Alberta, formed the basis for this report. The chicks were started in batteries, placed on range at 7 and 8 weeks, brought into the laying houses at 21 weeks, and kept there until the birds reached an age of 500 days. Slightly larger numbers than it was proposed to raise were started. The numbers were reduced by removing undetected males and a few others at 3 to 7 weeks after hatching. The numbers of chicks started after this adjustment were 2844, 3838 and 2760 in 1956, 1957 and 1958 respectively.

To obtain 0 to 500 day mortality, birds dying before they were placed on range, were assigned to either of the two ration groups on the basis of wing bands. This manipulation did not result in any serious error. In the case of the 1958 hatched birds, only the mortality up to the time they were placed in the laying houses is presented here.

LABORATORY PROCEDURES

Routine bacteriological examinations were made on organs of all dead birds. The livers were cultured on blood agar and sections of the duodenum were placed into tetrathionate or

selenite broth followed by incubation at 37 C and plating on MacConkey agar. Tissues were taken for histology from most cases of neoplastic disease. Hemagglutination-inhibition (HI) tests for Newcastle disease virus (NDV) and pleuropneumonia-like organisms (PPLO) were also made. The Connaught antigen* and technique were used for the latter. Some transmission experiments were made in chicks and embryonated eggs.

RESULTS

Overall mortality. The mortality occurring in the full-fed and restricted groups is shown in table 1. Deaths accounted for 13 percent and 16 percent of the full-fed group, and 19 and 20 percent of the restricted group for the 0 to 500 day period for the years 1956 and 1957 respectively. In the 1958 started birds, a high incidence of mortality occurred in the 147 day period in birds which were on the restricted ration, largely due to an outbreak of blackhead.

Leucosis and tumors. We follow Campbell[1] in excluding neurolymphomatosis from the leucoses; however, no cases of this condition were encountered in this survey. Neurolymphomatosis has been seen only infrequently at this laboratory. Leucotic and tumor conditions occurred at the low level of 0.6 percent (1956) and 1.1 percent (1957). The ration seemed to have exerted little influence. The observation period for the 1958 birds did not include the period over which losses from this cause usually occur. A list of the tumors encountered is given in table 2; the age incidence and distribution in organs in table 3 and 3a; as well as some illustrations in figures 4 to 10.

Seventy-five percent of the neoplastic conditions histologically examined were leucotic in nature. Nine cases of erythro- and myeloid leucosis occurred, many of which presented features of both conditions. Transmission experiments have been made in chicks with material from some of the cases of neoplastic disease but the results are not as yet available.

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TABLE I

Analysis of mortality

Year	Observation period in days	Ration	No. started	Leucoses: tumors		Reproductive disorders		Cannibalism		Specific Infections		Miscellaneous & undiagnosed		Total mortality	
				No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
1956	0-500	Full-fed	1422	10	0.7	35	2.5	49	3.5	20	1.4	71	5.0	185	13.0
		Restricted	1422	8	0.6	23	1.6	80	5.6	38	2.7	127	8.9	276	19.4
		Both	2844	18	0.6	58	2.0	129	4.5	58	2.0	198	7.0	461	16.2
1957	0-500	Full-fed	1919	24	1.3	68	3.5	89	4.6	9	0.5	116	6.1	306	16.0
		Restricted	1919	17	0.9	44	2.3	89	4.6	34	1.8	196	10.2	380	19.8
		Both	3838	41	1.1	112	2.9	178	4.6	43	1.1	312	8.1	686	17.9
1958	0-147	Full-fed	1380	2	0.1	0	0	4	0.3	5	0.4	21	1.5	32	2.3
		Restricted	1380	0	0	0	0	7	0.5	53	3.8	84	8.7	144	10.4
		Both	2760	2	0.1	0	0	11	0.4	58	2.1	105	3.8	176	6.4

TABLE II

Histological types of tumor and leucotic conditions encountered

Tumor	Year birds started		
	1956	1957	Total
Leucosis			
a) erythro-	2	1	3
b) myeloid	0	1	1
c) erythro-myeloid	3	2	5
d) lymphoid	6	22	28
e) myelocytoma	1	0	1
Sarcoma	3	3	6
Fibroma	0	1	1
Hemangioma	1	1	2
Other tumors	1	3	4
Total	17	34	51

A difficulty encountered in the survey was the histological characterization of the tumors. One tumor that caused difficulty was a solitary myelocytoma in the liver (figure 10) which might have been classed as an inflammatory rather than a neoplastic lesion. The figures for the incidence on lymphoid leucosis may be higher than they should be for some pathologists have a tendency to place under this group most undifferentiated round cell tumors. In figure 9, such a tumor is shown which we have termed lymphoid leucosis but, where, the high cytoplasmic-nuclear ratio makes this diagnosis suspect; in view of its ovarian origin it could be a highly anaplastic carcinoma.

Reproductive disorders. Under reproductive disorders were included egg peritonitis, impacted oviduct, prolapsed oviduct, etc. Losses from these disorders in 1956 and 1957 started birds were greater than losses from leucosis and neoplasia. Losses from reproductive diseases amounted to 2.0 percent and 2.9 percent in 1956 and 1957 started birds respectively. A higher mortality from these causes occurred in birds which had been given the full ration. The majority of deaths occurred after 350 days of age.

Cannibalism. Significant losses from cannibalism occurred in the 1956 and 1957 started birds. In 1956 there were more deaths in birds on the restricted diet; however, this difference was not maintained in the 1957 group. Deaths from cannibalism were most frequent in birds between 150 and 250 days of age.

TABLE III

Tumors and leucosis in 1956 started birds

No.	Age at death	Tumor	Primary site	Metastasis to other sites	Remarks
1	72	Hemangioma	Liver	Skin	Fibromatous reaction. Death from hemorrhage from skin
2	86	Erythroblastosis	Liver	Spleen	
3	114	Lymphoid leucosis	Liver	Ovary	
4	153	Lymphoid leucosis	Liver	Spleen	Atypical erythroblasts; many myelocytes present. May be stem-cell leucosis
5	333	Lymphoid leucosis	Liver	Spleen and bone marrow	Diffuse type; cells resembled mature lymphocytes (fig. 5)
6	335	Histiocytic sarcoma	Sternum	Lungs and muscle	Extensive areas of caseous necrosis
7	343	Lymphoid leucosis	?	Heart, kidney, pancreas, duodenum	Diffuse form: cells resembled lymphoblasts and lymphocytes
8	383	Erythromyeloblastosis	Liver	Spleen and bone marrow	Lesion in liver principally erythroblastic: marrow myeloblastic (fig. 6)
9	388	Lymphoid leucosis	Liver	Spleen	Cells resembled lymphoblasts
10	421	Sarcoma	Ovary	Wall of intestine and peritoneum	Tumor extremely vascular, could have been termed hemangioma
11	434	Sarcoma	Pelvic periosteum	None	At autopsy myelocytoma was diagnosed but histology showed it to be a-mixed-cell sarcoma
12	436	Lymphoid leucosis	Liver	Spleen and bone marrow	Diffuse type
13	448	Erythroblastosis	Liver	Spleen	Spleen many times the normal size (fig. 8)
14	458	Leiomyoma	Oviduct	None	Single tumor
15	474	Erythromyeloblastosis	Liver	None	Spleen was small and fibrotic
16	499	Erythromyeloblastosis	Liver	Bone marrow	Marrow replaced by cells of granulocytic series and with bone encroachment (fig. 7)
17	361	Myelocytoma	Liver	None	Tumor nodules composed of myelocytes, but may be a granuloma (fig. 10)

TABLE IIIa

Tumors and leucosis in 1957 birds

No.	Age at death	Tumor	Primary site	Metastasis to other sites	Remarks
1	65	Fibrosarcoma	Ovary	None	May be benign form of histiocytic sarcoma (fig. 4)
2	174	Adenoma	Kidney	None	The tumor which was the size of an orange, on section resembled normal kidney tissue
3	179	Myeloid leucosis	Liver	Ovary	The liver was grossly enlarged with miliary tumors which on section were seen to be myelocytes with mature granulation
4	189	Lymphoid leucosis	Proventriculus and duodenum	Intestine, ovary and adrenals	Tumor involvement of rectum and right ureter caused intestinal and urinary obstruction
5	243	Lymphoid leucosis	Liver	None	Spleen showed developing fibrosis and infiltration with granulocytes
6	246	Lymphoid leucosis	Liver	Ovary	Ovarian tumor growth suggestive of pseudorosette formation. May be a granulosa-cell tumor and not leucosis
7	261	Fibroma	Gizzard	Duodenum	Benign appearance belied by spread to duodenum
8	266	Lymphoid leucosis	Liver	Bursa of Fabricius	Growth in liver of nodular type; histologically diffuse. Kidney normal at autopsy but histologically infiltrated
9	271	Lymphoid leucosis	Liver	Proventriculus and viscera; bone marrow	Macroscopically leucosis of nodular type, but histologically also diffuse infiltration
10	280	Lymphoid leucosis	Ovary	Liver	Liver contained only early metastatic lesions
11	281	Lymphoid leucosis	Ovary	Liver	Liver contained only early metastatic lesions

TABLE IIIa (Cont'd.)

No.	Age at death	Tumor	Primary site	Metastasis to other sites	Remarks
12	285	Lymphoid leucosis	Liver	Ovary	Tumor cells resemble lymphoblasts; fibrous reaction to the cells in the liver, matched by firm nodules at autopsy
13	287	Lymphoid leucosis	Liver	Ovary and pericardium	Diffuse type
14	308	Erythroblastosis	Liver	Spleen	Typical erythroblastosis
15	312	Erythromyelo-blastosis	Liver	Spleen	Liver and comb showed yellow coloration suggesting associated jaundice
16	312	Lymphoid leucosis	Ovary	None	Diffuse type
17	315	Lymphoid leucosis	Kidney	None	Tumor restricted to upper lobe of right kidney
18	333	Lymphoid leucosis	Right lung	None	Diffuse type
19	338	Lymphoid leucosis	Ovary	Kidney	Diffuse type (fig. 9)
20	387	Lymphoid leucosis	Liver	Heart and lung	Cells resembled mature lymphocytes; nodular infiltration
21	389	Lymphoid leucosis	Pelvic region	Pancreas and liver	The origin of the pelvic tumor 3½" across was not certain; cells resembled lymphocytes, could be termed lymphoma or lymphocytoma
22	424	Hemangioma	Kidney	None	Tumor produced a cystic condition of all lobes of right kidney
23	438	Erythromyelo-blastosis	Liver	Spleen	

TABLE IIIa (Cont'd.)

No.	Age at death	Tumor	Primary site	Metastasis to other sites	Remarks
24	447	Lymphoid leucosis	Liver	Spleen	Cells at various stages of differentiation, from lymphoblasts to cells resembling lymphocytes
25	450	Not certain	Kidney	None	Cells primitive and difficult to determine origin; probably a lymphoma but extensive cytoplasm suggested epithelial origin; possible hypernephroma
26	451	Lymphoid leucosis	Duodenum	Liver	Many myelocytes seen in association with lymphoid infiltration
27	454	Lymphoid leucosis	Liver	Spleen	
28	456	Granulosa	Ovary	None	Tumor was brain-like in appearance, grayish-red, 2" across
29	471	Lymphoid leucosis	Liver	Spleen	Small round cell infiltration
30	489	Lymphoid leucosis	Liver	Spleen	Infiltrations by both blastic cells and small round cells; asociated hepatitis
31	500	Lymphoid leucosis	Liver	Spleen	Infiltration by both blastic cells and small round cells; associated hepatitis
32*	313	Fibrosarcoma	Ovary	Pectoral muscle	Caseation in pectoral lesions
33*	340	Lymphoid leucosis	Duodenum	None	Associated egg peritonitis
34*	352	Histiocytic sarcoma	Heart	Liver	Associated egg peritonitis

* Associated conditions possibly the cause of death.

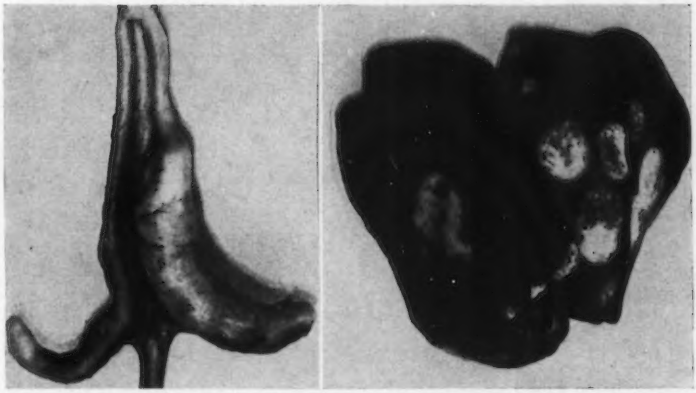

Fig. 1. (left) and 2. (right) Liver and ceca from cases of "blackhead" in 1958 started birds.

Specific infections. Under specific infections, we have included losses from coccidiosis, blackhead and chronic respiratory disease (CRD), which accounted for a high mortality each year. Twenty-five cases of CRD occurred in the full-fed group and forty cases in the restricted group, showing a bias in favor of the latter. Both cecal and intestinal coccidiosis were recognized. These occurred at a very low level.

Blackhead caused serious losses in the birds on the restricted diet; the cecal and liver lesions are illustrated in figures 1 and 2 and the histopathology in figure 3. Blackhead was encountered in the 1956 and 1957 birds but no histological examination was made. However, early in 1958 we noted that Margoliash[4] in Denmark had described a similar condition which he believed was due to some inadequacy of the diet. This made us question our earlier diagnoses. However, histological sections made on subsequent cases showed the presence of histomonads (figure 3).

Miscellaneous conditions. We have classified as miscellaneous conditions: fatty degeneration of the liver, massive liver necrosis, colibacillosis, a few cases of staphylococcosis, two cases of salmonellosis, hemorrhagic disorders, suffocation,

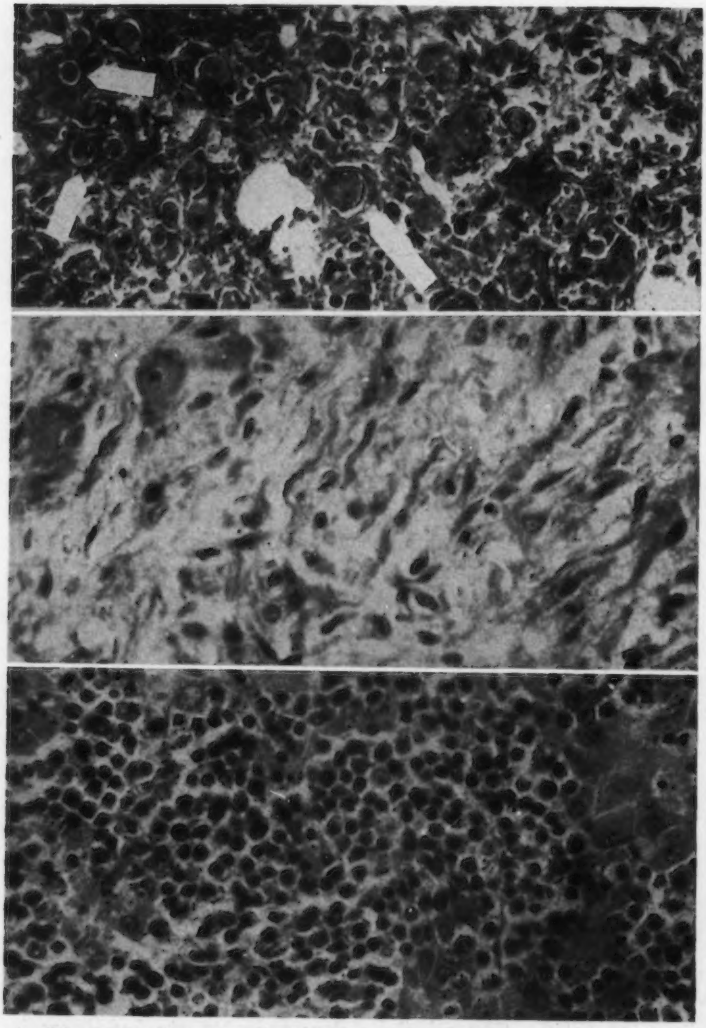

Fig. 3. (top) Histomonads in necrotic liver tissue. H and E stain x 700.

Fig. 4. (center) Fibrosarcoma of ovary; bird 1 (1957-58). H and E stain. x 1100.

Fig. 5. (bottom) Lymphoid leucosis, liver; bird 5 (1956-57). H and E stain. x 1100.

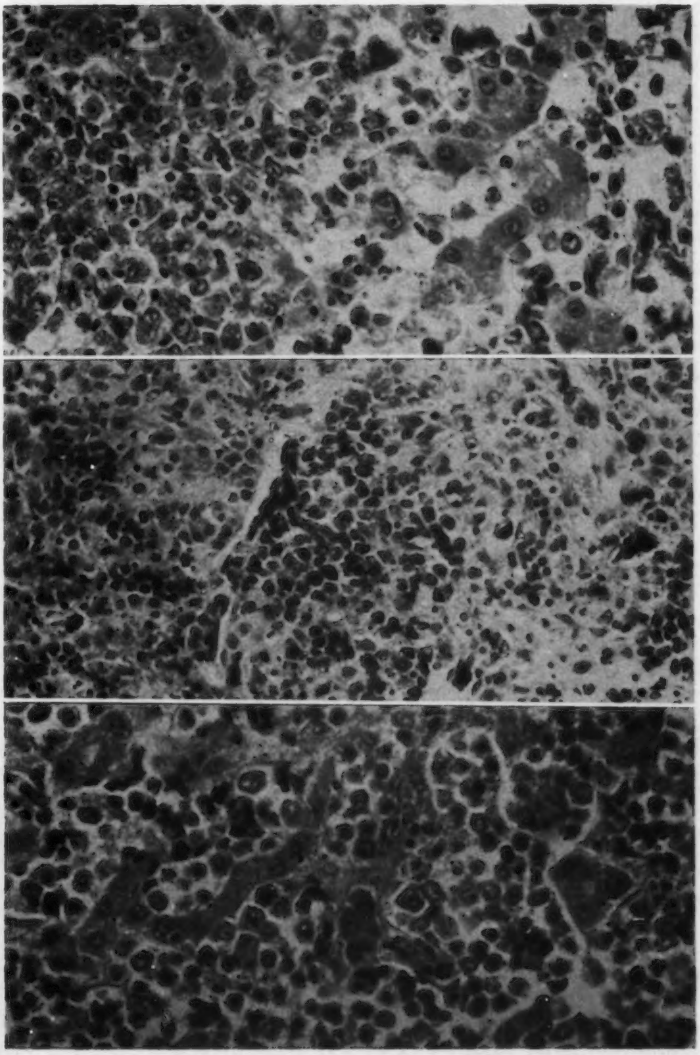

Fig 6. (top) Erythromyeloblastosis, liver; bird 8 (1956-57) .Forms of myelocytes to left, erythroblastic cells in sinusoids to right. H and E stain. x 1100.

Fig. 7. (center) Myeloblastosis, liver; cells of type found in myelocytoma; bird 16 (1956-57). H and E stain. x 700.

Fig. 8. (bottom) Erythroblastosis, liver; bird 13 (1956-57). H and E stain. x 1100.

accidental trauma and drowning, etc., and cases for which the cause of death was not ascertained. Table 1 shows greater losses under this heading in the restricted birds than in the controls.

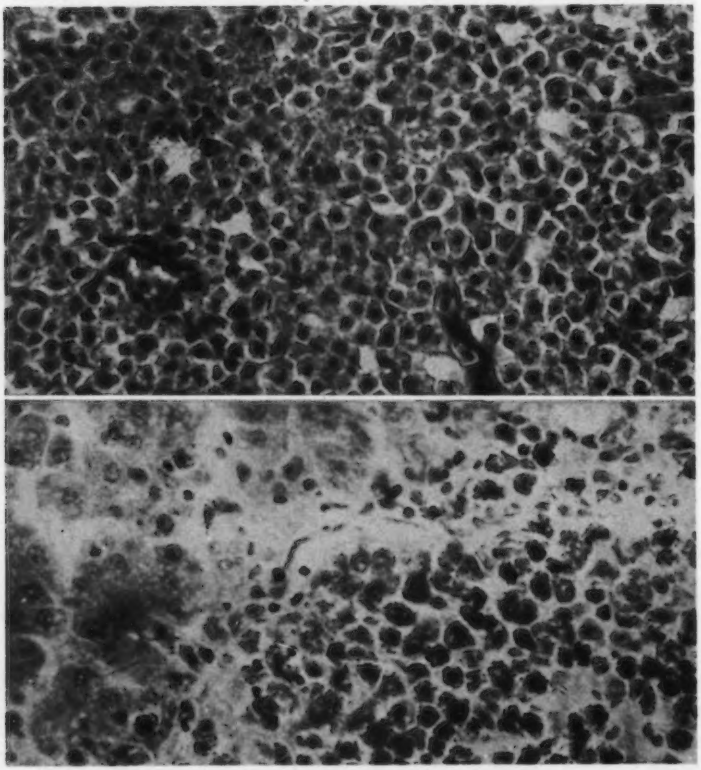

Fig. 9. (top) Ovarian tumor, diagnosed as lymphoid leucosis; bird 19 (1957-58). H and E stain. x 1100.
Fig. 10. (bottom) Myelocytoma in liver; bird 17 (1956-57). H and E stain. x 1100.

Transmission experiments. Transmission attempts were made in other instances as well as with neoplastic tissues, including an attempt to transmit nephritis recognized in a 1957 started bird which died at 412 days of age. A hemagglutinat-

ing virus was obtained which was apparently NDV on the basis of HI test; however, ninety-six sera from 1957 started birds bled after this isolation, were negative to HI tests. Materials saved from 1957 birds dying from neoplastic conditions did not yield NDV in egg transmission experiments. The origin and importance of this virus was, therefore, not clear.

Because of the suspected occurrence of CRD in the flock, HI tests for PPLO were carried out on two occasions. If inhibition titers greater than 1:20 are accepted as positive, 23 out of 24 sera collected in February 1957 from the 1956 started birds gave positive titers. Positive titers were not obtained in 36 sera from young birds from our own two flocks which were included as controls. Sera were collected from the 1957 and 1958 started birds. Equal numbers of samples were taken from birds on the full and restricted feed groups. Ninety of 100 sera from the 1957 started birds gave positive titers. In the 1958 started birds, 50 sera from the full-fed group were negative, while only 3 of 50 sera were negative in the restricted group. The latter observation was difficult to explain since CRD was clinically recognized in both of these groups.

ACKNOWLEDGMENTS

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SUMMARY

Birds dying out of a total of 9442 white Leghorns over a 3 year period were examined at the laboratory. Half the birds which were maintained on a restricted diet for part of the growing period had a mortality of 19 and 20 percent respectively for 2 years. The mortality in the full fed group was 13 and 16 percent.

Blackhead occurred at a much higher level in birds on the restricted feed but it is not clear whether this was due to dietary or stress factors.

Losses from neoplastic disease in general and leucosis in particular were very low in this survey,[2] but it should not be inferred that these conditions exist at a similar low level in

flocks throughout Canada. Since the level of these diseases varies significantly from flock to flock and from year to year,[3] the low incidence may only mean that the resistance of these birds was at a fairly high level over the period of the study.

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CONJUNCTIVITIS OF CHICKENS CAUSED BY A TYPICAL INFECTIOUS LARYNGOTRACHEITIS VIRUS

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SATRIANO *et al.*[2] reported the isolation of a mild srain of infecious laryngotracheitis (ILT) from the lacrimal fluid of 19-day-old Cornish Cross Chicks which did not show respiratory signs. In 1957, several field reports from southern California indicated the presence of an unusual hemorrhagic conjunctivitis affecting birds of different ages. Both broilers

and laying flocks suffered losses exceeding, in a few instances, 50 percent of the affected birds. Occasionally, conjunctivitis was observed in flocks recently vaccinated against infectious laryngotracheitis (ILT). In some instances, no other signs but conjunctivitis were reported.

In the acute and the recovery stages, a retraction of the lids would give the eyes a slanted appearance generally referred to as almond-shaped eyes. Lacrimation was common Secondary bacterial infection often resulted in occlusion of the eyelids.

EXPERIMENTAL

A representative sample from an affected flock of 2-month-old White Leghorns was obtained. The lacrimal fluid was aspirated into a sterile pipette and, after addition of 1,000 units of penicillin and 1,000 micrograms of streptomycin was inoculated into six 8-day-old embryonating chicken eggs. The eggs were inoculated according to the method of Fabricant.[1] The only variation was that approximately one-half the inoculum was deposited on the chorioallantoic (ca) membrane. Sterility tests indicated the absence of bacteria in the inoculum. After incubating the eggs at 36 C, one embryo was dead on the 4th and two on the 5th day after inoculation. An examination of the ca membranes of the dead embryos revealed light grey plaques. These membranes were aseptically removed, ground in a sterile mortar with the ca fluid of the same eggs, and passed four consecutive times into six 10-day-old embryonating eggs. At the 4th passage, the membranes were harvested aseptically, suspended in the ca fluid and frozen. A portion of the ca fluid was tested for hemagglutination activity typical of Newcastle disease virus. The membranes were thawed, ground, and brushed into 20 feather follicles of two susceptible and two fowl-pox immune birds. At the same time 20 2-month-old susceptible White Leghorns were vaccinated intracoacally with the mixture. The next day another group of 20 2-month-old susceptible White Leghorns were vaccinated intracloacally with a commercially available ILT vaccine, containing 10^5 plaque-forming units per ml of the reconstituted vaccine.

Three weeks after vaccination of both groups, 10 susceptible birds of the same source and age were added to each of the two groups. At that time the group vaccinated with the commercial ILT vaccine and the 10 controls were inoculated intratracheally with 0.25 ml of the ca membrane suspension. Each bird received approximately 2,500 plaque-forming units of the unknown agent. The group vaccinated with the membrane suspensions and the 10 controls were inoculated intratracheally with 0.25 ml of the reconstituted commercial vaccine. Each bird received approximately 25,000 plaque-forming units of ILT virus.

The birds were observed daily for respiratory symptoms. The third day after inoculation, some of the control birds in each group showed gurgling and gasping. Three birds from each group were killed on the 5th day after inoculation and intranuclear inclusion bodies typical of ILT were observed in the epithelial cells of the tracheal mucosa. None of the vaccinated birds showed either respiratory symptoms or intranuclear inclusion bodies.

The hemagglutination test for Newcastle disease virus was negative. Lesions typical of fowl pox were not observed in either one of the four birds brushed with the ground membranes. Eight days after intratracheal inoculation of the controls with the ca membrane suspension, hemorrhagic conjunctivitis was observed in four of them. Histological sections of the conjunctival mucosa showed presence of intranuclear inclusion bodis typical of ILT.

CONCLUSIONS

It can be concluded, therefore, that ILT virus was responsible for the hemorrhagic conjunctivitis. Furhtermore, the ILT virus isolated from the lacrimal fluid of the original birds was antigenically similar to a commercially available ILT vaccine. Previous experimental work with this vaccine had shown its antigenic similarity with other vaccine and field strains of ILT virus.

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INGESTION OF *AMMI VISNAGA* SEEDS AND PHOTOSENSITIZATION— THE CAUSE OF VESICULAR DERMATITIS IN FOWLS

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VARIOUS investigators have studied a disease in fowls characterized by the formation of vesicles which once ruptured are replaced by crusts found on the tips of the comb, face, wattles, feet, and toes. These manifestations, similar to those in foot and mouth disease of susceptible species, were also observed in turkeys, ducks, and geese. Becker[1] and Van Heelsbergen[13] were not able to demonstrate any relationship between these lesions and the virus of foot and mouth disease. Hoffman[6] called this syndrome vesicular dermatitis. From the fluid obtained from the lesions, he isolated a staphylococcus with which he succeeded in reproducing the clinical picture. There was also a marked decline in production and mortality of 10 percent.

Perek[11] studied vesicular dermatitis and reproduced the condition by feeding healthy chickens seeds of *Lolium temulentum* which were contaminated with *Cladosporium herbarum*. Cassamagnaghi and Cassamagnaghi, Jr.[3] succeeded in establishing that the seeds of *Ammi visnaga* administered via the digestive tract, were able to reproduce all of the symptoms when the animals were exposed to sunlight afterwards. Other plants have similar effects on various species of animals.

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Hertwing[5] encountered a narcotic like action in *Fagopyrum esculentum*. This phenomenon was confirmed by Merian,[10] Lutz, and Schmid[7] and Mathews.[8] These investigators called this disease fagopyrism. To the condition produced by *Hypericum perforatum*, they gave the name hypericism and the term geeldikop to a disease in South Africa probably caused by photosensitization.

Clare[4] studied photosensitization in New Zealand and Mathews[8] studied it in Texas and New Mexico in the United States and in Mexico. This investigator established that part of the symptoms produced by the ingestion of *Agave lechuguilla*, a plant which is encountered in these regions, was due to photosensitization. There is suspected without definite evidence the existence of sensitizations produced by other plants such as alfalfa (*Medicaga sativa*), clover (*Medicaga denticulata*), peas (*Vicia sp.*) etc.[2]

VESICULAR DERMATITIS PRODUCED BY *AMMI VISNAGA*

In the poultry department of the Veterinary College and in the poultry pathology service of the Dr. Miguel C. Rubino Laboratories of Animal Biology, we have studied the effect of biznaga (*Ammi vinsnaga*) (figure 4) for the last seven years. The lesions produced by this photosensitization which are identical to the descriptions of other vesicular dermatidites, begin by small vesicles which later become confluent to form large vesicles (figure 1) and which are first observed on the comb, wattles, face, eyelids, and later on the legs and toes. After a few days the vesicles break and exude a clear fluid turning to a yellowish or orange color (figure 2). The blepharitis which is observed in some cases causes mechanical interference with vision. The lesions on the feet cause loss of the superficial scales and an almost complete sloughing resulting in open ulcers. This lesion is even more serious between the toes. The birds remain dumpy and can move only with great difficulty.

In ducks, the disease begins on the beak with vesicles which, after breaking, leave a hard scab (figure 3). On the legs, toes and web of the feet, vesicles are also produced followed by scabs.

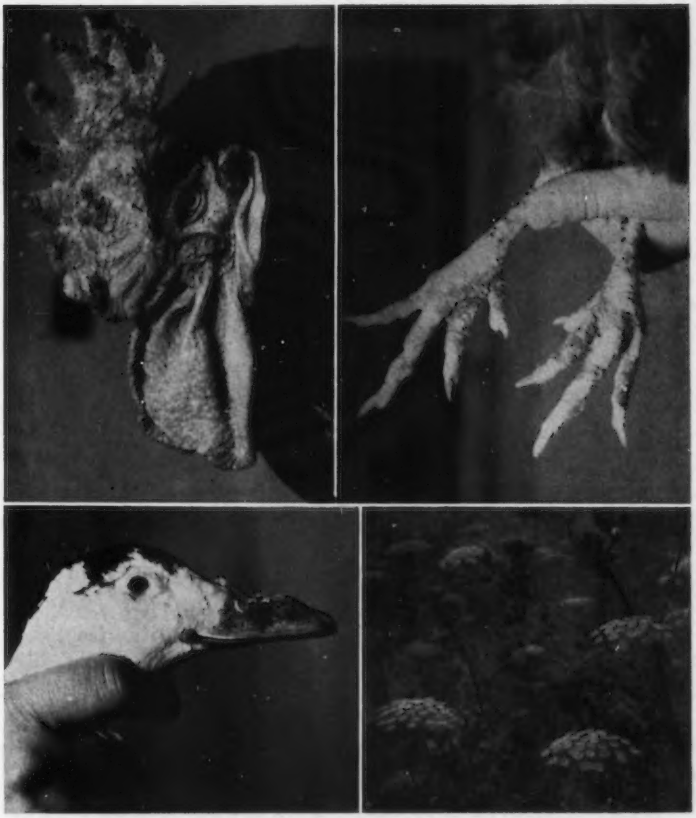

Fig. 1. (upper left). Head of bird with vesicles and scabs typical of vesicular dermatitis caused by ingestion of *Ammi visnaga* seeds and exposure to sunlight.

Fig. 2. (upper right). Feet and toes of affected bird with lesions of vesicular dermatitis.

Fig. 3. (lower left). Lesions on the upper beak of a duck caused by ingestion of *Ammi visnaga* seeds and exposure to sunlight.

Fig. 4. (lower right). The plant *Ammi visnaga* as it grows in the field.

The birds recover in 3 to 4 weeks after the visnaga is eliminated from the ration. During the disease, egg production is greatly lowered. Necropsies of birds with typical lesions of vesicular dermatitis, do not reveal any internal lesions. In all cases, examination of the ration employed in feeding the birds, a mixture of ground grains, wheat, and bran, seeds of biznaga were always found. On a breeding farm where wheat mixed with biznaga was accidentally used for feeding the birds, vesicular dermatitis was observed in all of the adult fowls. From each sack of 50 kilos of wheat we screened out on the average 1300 grams of visnaga seeds.

EXPERIMENTAL

To obtain evidence that seeds of visnaga play a part in the etiology of vesicular dermatitis, we carried out the following experiments. Six lots of 50 chickens each were given the following rations:

Group 1, biznaga seeds recovered by screening the sacks of wheat found on the breeding farm. The seeds were mixed in a regular ration.

Group 2, wheat from which the biznaga seeds had been screened out.

Group 3, wheat mixed with biznaga collected in the field.

Group 4, pure visnaga collected in the field.

During the experiment these four groups of birds were kept outdoors, exposed to sunlight.

Group 5, biznaga mixed in our regular poultry ration used in our laboratory.

Group 6, wheat and biznaga found on the breeding farm.

Groups 5 and 6 were maintained indoors during the experiment and for the most part protected from exposure to the sun's rays.

The disease was reproduced with symptoms identical to the natural vesicular dermatitis in all the birds of groups 1, 3 and 4. The appearance of lesions of the disease occurred in 3 to 5 days in every group. No birds were unaffected. No abnormalties were seen in lots 2, 5 and 6. On autopsy, the sick birds

had nothing abnormal aside from the external lesions.

Inoculations of the usual laboratory culture media and chicken embryos were made with the vesicles and the scabs. Staphylococci and other organisms were isolated in the bacteriological culture media. Inoculation of these same organisms into chickens by the subcutaneous or intramuscular route produced no change other than some local reactions. Inoculation of scabs suspended in physiological salt solution by scarification of combs and wattles was done in ten birds. No changes were noted in these inoculated fowls one month after the test. Attempts were made to isolate a virus by the inoculation of the chorio-allantoic membrane of 9-day-old chick embryos and also by the inoculation of suckling mice for the purpose of demonstrating the presence of foot and mouth disease virus. All of these attempts failed.

ACKNOWLEDGMENTS

The assistance of Dr. P. P. Levine in translating and preparing this paper for publication is gratefully acknowledged.

CONCLUSIONS

Vesicular dermatitis studied by us was not caused by any bacterial or viral infectious agent. Inoculations by the subcutaneous and intramuscular route and by scarification of skin done with suspension of scabs did not prove the presence of a transmissible disease.

On the other hand, feeding trials showed that vesicular dermatitis appeared when seeds of biznaga were fed. The pure wheat that had been cleaned of the seeds by means of a screen was shown to be completely safe.

Another factor is necessary to produce the disease, namely, the action of sunlight. This was proven when birds fed with biznaga but maintained in the shade did not come down.

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ATYPICAL PASTEURELLA INFECTIONS IN CHICKENS*

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FOR several years atypical Pasteurella have been encountered in cultural studies of chickens with respiratory infections submitted to the Livestock and Poultry Pathological Laboratory, San Gabriel, California. As a result, the

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infection has become recognized as a specific disease in Southern California. To differentiate the condition from fowl cholera and infectious coryza, it has become known among poultrymen by the name of Pasteurellosis or Pasteurella infection. All evidence indicates that these organisms play a major part in the respiratory disease complex which plagues many poultrymen in southern California.

Hall *et al.*[1] in 1955 described a new species of Pasteurella encountered in chronic fowl cholera. The organism they described was of low pathogenicity. Out of many chickens and rabbits inoculated, only occasionally did death occur. These bacteria were isolated consistently more often than *Pasteurella multocida* from live chickens in flocks suffering from chronic fowl cholera, as well as from flocks suffering from undiagnosed disorders usually initiated with a coryza. It was of special interest that the organisms had not been isolated from outbreaks of acute fowl cholera where the disease was not accompanied by coryza. The bacteria were not isolated from healthy fowls, but always appeared in association with other maladies characterized by an inflammation of the upper respiratory tract. Cross agglutination studies indicated that the organism is serologically distinct from *Pasteurella multocida* and *Pasteurella hemolytica*. The authors proposed that this species of Pasteurella be named *Pasteurella gallinarum*.

A special study of Pasteurella infections was conducted in the Demler Farms Pathological Laboratory, Anaheim, California, during the year 1959. It was found that two biochemically different types of Pasteurella, other than *Pasteurella multocida*, were primarily involved. One of these was apparently identical to that which Hall *et al.*[1] proposed to be called *Pasteurella gallinarum*, and will appear here under that name. The other organism, to the best of our knowledge has not been previously described. The biochemical characteristics indicate that it may be one of the three atypical group 7 poultry strains of Pasteurella described by Schneider.[4] This new organism for the purpose of identification will be designated as *Pasteurella n.sp.* or *P. n.sp.* in this paper.

During the one year study, at least one of these atypical Pasteurella were isolated from 113 groups of birds out of 242 groups of chickens examined bacteriologically (46.69%). The infected flocks represented 1,800,000 White Leghorns and White Leghorn-cross egg producing chickens.

Heddleston,[3] to whom cultures were submitted, stated that both of our organisms were serologically similar to *Pasteurella gallinarum* by the agglutination test. He also observed that the strains we sent him were more virulent than those which he had previously studied. As has been our experience, he also noted that our two organisms produced mortality in test chicks inoculated intramuscularly whereas neither caused signs or lesions in chickens when inoculated intranasally or into the palatine cleft.

It appears that the organism which we identify as *Pasteurella n.sp.* produces a more severe and usually more generalized type of infection than does *Pasteurella gallinarum*. However, it has been our observation that both strains of bacteria produce similar lesions, and to date we have been unable to differentiate the two infections in chickens by signs or pathological manifestations. For this reason in describing the disease as it appears in the field and the pathology produced, the terms "Pasteurellosis" or "Pasteurella infection" will be used to indicate either a *Pasteurella gallinarum* or a *Pasteurella n.s.p.* infection. *Pasteurella multocida* infections will not be included in this discussion, although it is occasionally found to be involved.

Preliminary inoculation trials in this laboratory have indicated that the disease is harmful principally as a complication or secondary disease. When cultures of the bacteria were administered intranasally or in the drinking water to healthy, susceptible chicks, no perceptible sickness or pathology developed. On repeated occasions, chickens in the respiratory phase of Newcastle disease or infectious bronchitis vaccination reaction, were artificially exposed through the respiratory tract to either *Pasteurella gallinarum* or *Pasteurella n.sp.* No evidence of complications as seen in the field developed. When

these organisms were inoculated, in combination or singly, simultaneously with *Hemophilus gallinarum* into the nostrils or palatine cleft of chicks, a much more severe sinus infection occurred than in young birds inoculated in the same manner with *H. gallinarum* alone. When either of the Pasteurella were inoculated in combination with *H. gallinarum* into the sinuses of chickens in the respiratory phase of laboratory induced infectious bronchitis or Newcastle disease, severe complications with internal lesions resembling those seen in the field developed. In the presence of PPLO, the condition becomes even more serious.

On several occcasions one of the atypical Pasteurella has been isolated from apparently healthy chickens of all age groups, from ranches where the disease is known to exist. These isolations were made from the conjunctiva, anterior chamber of the eye, and sinuses. This would suggest that both organisms can be present without causing any evidence of sickness, and would further substantiate evidence that they are opportunists rather than primary infection types of bacteria.

ATYPICAL PASTEURELLA INFECTION

The field disease. Field observations offer evidence that the severity of the infection is directly related to concurrent infections as well as to various management and environmental factors which produce stress or lowered vitality. The disease is a particular problem where frequent brooding of replacement pullets is practiced, each brood transmitting the infection to successive groups of younger highly susceptible growing chickens. On most of these ranches, infectious coryza appears to be a primary inciting agent. It has been our experience that bacteriological cultures from sinuses of chickens in the first few days of an outbreak often yield pure *H. gallinarum*, whereas a week later only Pasteurella can be isolated from the same flock. In other instances, the infection appears to occur in association with Newcastle disease, infectious bronchitis, laryngotracheitis, or chronic respiratory disease, without the involvement of *H. gallinarum*. However, it is our opinion that *H. gallinarum* may have been involved in these

instances but we were unable to recover it.

Pasteurella infections are seen almost exclusively among young, growing birds, occurring most frequently when chicks are moved out of the brooders to the growing area. On crowded ranches, however, where brooders are close to grow areas, very young chicks may become infected. The disease usually persists through the growing age, gradually diminishing as the chickens reach maturity. Adults rarely show signs after going through the disease during the growing period.

In many instances, the disease first appears in an acute form, which later develops into a chronic infection of long duration. In almost every case in which the acute disease appears, a primary viral inciting agent in addition to *H. gallinarum* can be demonstrated. Newcastle disease and infectious bronchitis seem to be the most common primary infections. However, laryngotracheitis is often also incriminated.

In other instances, the infection appears gradually in a chronic form in association with infectious coryza, and continues in this manner throughout the growing period. In this form of the disease, sudden mortality is rare. Gradual losses which increase over a period of time occur, due usually to complications with CRD. In this type of infection, a primary viral agent can not usually be demonstrated. Primary inciting agents in this year's study are shown in table 1.

TABLE I

Inciting agents associated with *Pasteurella gallinarum* and *Pasteurella n.sp.* infections.

	No. of groups	Percent
Infectious Coryza	61	54.9
Newcastle Disease	23	20.3
Newcastle vaccination reaction	5	4.4
Infectious Bronchitis	11	9.7
Bronchitis vaccination reaction	6	5.3
Laryngotracheitis	5	4.4
Pox vaccination reaction	1	.9
Negative for inciting agent	1	.9
Total groups studied	113	

In a typical, acutely infected flock, many birds suddenly appear very sick with ruffled feathers, extreme inappetance and inactivity. Paleness and rapid emaciation occur. The general appearance is similar to that of chickens affected with acute coccidiosis. Some individuals show a characteristic swelling of the intermandibular space and there are usually some with coryza-like swellings of the infraorbital sinuses. In many instances, particularly when Newcastle disease or infectious bronchitis is primarily involved, ruffled feathers, rapid emaciation, and heavy mortality are frequently the only signs displayed. Some birds may show a slow gasp, or may stand with beaks partially open. Mortality occurs suddenly to many chickens in excellent flesh during the acute stage of the disease.

Gross Pathology. Necropsy nearly always reveals a sinus infection. The exudate found in the sinuses is usually more liquid and transparent than the creamy white purulent material associated with the *Hemophilus gallinarum* infections. Edema and cellulitis of the intermandibular tissues is often present. As the infection progresses, an organized, light yellow, fibrinous mass develops in the subcutaneous tissues between the mandibles, frequently extending down the neck under the skin. A similar fibrinous yellow membrane may be found beneath the skin of the medial aspect of the femoral portion of the legs. Also very often, a fibrinous membrane is found beneath the skin covering the abdomen, with a cellulitis sometimes involving the muscles over the posterior portion of the sternum. Especially characteristic of the infection in acute or peracute stages is a marked pericarditis and perihepatitis. These lesions are very similar to those seen with acute colibacillosis. In acute Pasteurella infections, the color of the fibrinous material surrounding the heart, and over the liver is usually of a translucent, pearly white color. In our experience with colibacillosis, the color of this material is deep yellow and opaque. As a rule, in acute Pasteurella infections, the spleens and livers appear normal in size and consistency, and there is usually very little evidence of tracheal involvement.

In chronic infections, a mucoid sinusitis with no facial swelling and a severe mucoid tracheitis are characteristic. In this type of infection, PPLO can almost always be demonstrated along with Pasteurella.

Fig. 1. Ten-week-old chicken with acute generalized atypical Pasteurella infection. Arrows show characteristic fibrinous membranes over liver and pericarditis.

Prevention. The most successful method of preventing Pasteurella infections has been through avoiding primary viral infections or severe vaccination reactions, by using judicious immunization programs against Newcastly disease, infectious bronchitis, laryngotracheitis, and fowl pox.

In many instances, however, infectious coryza is apparently the only primary agent. Prevention of infectious coryza by correction of management factors to minimize contact between infected carriers and susceptible younger age groups has shown good results. Studies of living and killed *Hemophilus gallinarum* vaccines are at present in process.

During the past year, more than 100,000 growing pullets were vaccinated with killed autogenous Pasteurella vaccines using the two strains of bacteria, singly or in combination. In

most cases, oil emulsion type bacterins prepared in the manner described by Heddleston[2] were used. The results of these trials have thus far proven disappointing, but further studies are in process. Pasteurella multocida bacterins have shown no value in protecting against these two species of organisms.

Drug prophylaxis and therapy. Many sulfonamides, antibiotics, and nitrofurans have been used in attempts to combat the disease. The sulfonamides have proven far superior both in vitro and in vivo. In most instances, sulfaquinoxoline has been the most effective. This drug has also been helpful in preventing the infection when continuously carried in the feed at the coccidiostatic level of .0125 percent.

In acutely infected chickens, injectable sulfamethazine often gives good results. Antibiotics, particularly the tetracyclines, are of value as supplementary treatments, especially in instances where PPLO is involved.

Bacteriology. Most frequent isolations were made from the sinuses, pericardium, or from beneath the fibrinous membrane which is formed over the liver in acutely affected birds. Occasionally, recoveries of Pasteurella were made from the

TABLE II

Comparative frequency of isolations of *Pasteurella gallinarum* and *Pasteurella n.sp.* from various organs.

	Pasteurella gallinarum	*Pasteurella n.sp.*	Total
Sinus	64	40	104
Pericardium	3	20	23
Liver Surface	1	14	15
Liver	1	6	7
Spleen	0	6	6
Trachea	2	2	4
Subcutaneous	0	4	4
Wattle	3	0	3
Heart blood	0	2	2
Anterior chamber of the eye	1	1	2
Total isolations	75	95	170

caseous exudate beneath the skin of the neck, flanks, and breast. It is shown in table 2 that both organisms were most often recovered from the sinuses. *Pasteurella n. sp.* was much more frequently isolated from the pericardium and liver sur-

faces than was *Pasteurella gallinarum*. On five occasions, both organisms were recovered from the same group of birds.

Initial isolations of both organisms are greatly enhanced by using blood agar and microaerophilic conditions. It has been our experience that trypticase soy agar (BBL), enriched with 5 percent defibrinated rabbit blood gives best growth. Chicken blood was originally used but gave poor results. A candle jar has proven very practical for microaerophilic conditions.

On blood agar, the colonies usually are fluorescent, circular, smooth, entire, convex, and translucent. *Pasteurella n.sp.* usually produces a more mucoid and spreading colony than does *Pasteurella gallinarum*. There is usually relatively good growth on 5 percent serum infusion agar and serum dextrose starch agar. Growth on these media without serum has been poor, but is increased by using microaerophilic conditions. On repeated passages, *Pasteurella n.sp.* tends to lose its ability to grow on artificial media. We have had very poor success in propagating this organism without microaerophilic conditions. This has not appeared to be true of *Pasteurella gallinarum*. After initial isolation, the latter bacteria does not require

TABLE III

Biochemical characterization of *Pasteurella gallinarum* and *Pasteurella n.sp.*

	Pasteurella gallinarum	*Pasteurella n.sp.*
Arabinose	—	—
Xylose	—	—
Glucose	+	+
Levulose	+	+
Mannose	+	+
Lactose	—	—
Sucrose	+	+
Maltose	+	—
Trehalose	+	—
Dextrin	+	—
Mannitol	—	—
Dulcitol	—	—
Salicin	—	—
Hemolysis	—	—
Indol	—	—
Nitrate reduction	+	+
Gas formation	—	—

blood, serum, or microaerophilic conditions.

In this study, a fermentable broth (BBL infusion broth) consisting of 1 percent carbohydrates, alcohols, and a glucoside were used. Both species of Pasteurella grow well in this particular media without the use of serum, although *P. n.sp.* requires more time to ferment carbohydrates. Visual observations of acid production were determined by the use of 1 percent Andrade indicator without the aid of a pH meter. Complete biochemical reactions are tabulated in table 3. Differentiating biochemical reactions are shown in table 4.

TABLE IV

Differentiating biochemical characteristics of *Pasteurella multocida*, *Pasteurella gallinarum* and *Pasteurella n.sp.*

	P. multocida	*P. gallinarum*	*P. n.sp.*
Maltose	—	+	—
Mannite	+	—	—
Trehalose	—	+	—
Dextrin	—	+	—
Indol	+	—	—

Both organisms are gram negative, bipolar non-motile rods. The cells usually occur singly but occasionally are filamentous, especially *Pasteurella gallinarum*. *Pasteurella n.sp.* appears to be more pleomorphic and smaller with more coccoid or coccobacillus-like forms.

The serological relationship between *Pasteurella gallinarum and Pasteurella n. sp.* is still being studied.

SUMMARY

Two strains of Pasteurella, biochemically and serologically different from *Pasteurella multocida*, have become an important problem in the respiratory disease complex seen among southern California chickens. One of the strains appears to be the same as that described by Hall *et al.*[1] as *Pasteurella gallinarum*. The other is similar serologically, but different biochemically. The differences are described.

The infection appears to be chiefly secondary, most often associated with infectious coryza. It also is seen frequently

as a complication associated with other respiratory diseases, in which the presence of *H. gallinarum* could not be demonstrated. It is believed, however, that *H. gallinarum* may have been present prior to these other respiratory infections.

Prevention by avoiding primary virus infections, with a good vaccination program, and improvement of management factors to reduce the incidence of infectious coryza have been effective means of preventing the disease. Autogenous bacterins made from the two strains of Pasteurella or *Pasteurella multocida* vaccines have not been successful. Treatment with sulfonamides, particularly sulfaquinoxoline, has been effective. The continuous prophylactic level of .0125 percent suffaquinoxoline in the feed is of value in avoiding severe outbreaks.

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REVACCINATION PROCEDURES FOR INFECTIOUS LARYNGOTRACHEITIS*

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A PREVAILING impression among poultrymen regarding immunity against laryngotracheitis (LT) is that one vaccination confers lifelong protection to the bird. The validity of such a premise is lacking. Gibbs[1] demonstrated that LT immunity was not durable by infecting six of 55 birds 5 months after vaccination. Ranby[5] likewise refers to the transient nature of immunity when he states: "Immunity in chickens vaccinated very young wears off after a time." The occurrence of occasional outbreaks of LT in previously vaccinated flocks does not substantiate the claim for lifelong immunity. The following experiments were conducted in an attempt to improve immunity to LT by revaccination.

METHODS AND RESULTS

Vaccine. The vaccine used was taken from regular production lots of LT vaccine and reconstituted as recommended for field use.

Drop application. This consisted of dropping one drop of vaccine from a 19 gauge needle onto the vent, eye or nostril of the bird as indicated.

Vent brush application. This involved the use of a stiff bristled brush for applying the vaccine, dipping the brush in the vaccine, then making three to five brushing strokes across the mucous membrane of the everted cloaca.

Serum neutralization (SN) test. The SN test was per-

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formed by mixing constant amounts of serum with 10-fold dilutions of virus, incubating the mixtures for one hour at room temperature, then inoculating 10 or 11-day-old chicken embryos by the dropped membrane technique. Details of the test were described by Hitchner *et al.*[2]

Previous studies of the infraorbital sinus route of challenge[3] for LT had raised doubts about the duration of immunity following vaccination, and suggested the need for revaccination to enhance the protection against the disease.[4] To study this question further, an experiment was conducted comparing the brush and drop method of vaccine application to White Rock chickens of one, 4 and 8 weeks of age. Five nonvaccinated contact controls were placed with each age group 10 days postvaccination.

Each ampule of vaccine used for initial vaccination at the respective ages was titrated in 10 or 11-day-old embryos by the dropped membrane technique. The vent "take" readings were made on the 4th day postvaccination. The 50 percent embryo infective dose (EID_{50}) titer of the vaccine and the "take" readings are shown in table 1. The "takes" were graded as strong, weak or negative. Past experience has shown that if 50 percent or more of a group of birds show positive takes (strong or weak) to vaccination, the remainder of the group is resistant to challenge.[3] By this criterion, the vaccination was satisfactory in all groups.

TABLE I

Vent reaction to drop and brush methods of LT vaccine application at one, 4 and 8 weeks of age

Age vac.	Vaccine Titer (log_{10} EID_{50}/ml)	Method	No. birds	"Take" Readings		
				Strong	Weak	Negative
1 week	6.8	Drop	50	32	17	1
		Brush	50	31	18	1
4 weeks	5.6	Drop	25	8	11	6
		Brush	24	20	4	
8 weeks	6.6	Drop	25	16	9	
		Brush	25	24	1	

At 16 weeks of age, 10 to 15 birds from each of the vaccinated groups and 10 nonvaccinated controls were each challenged in one sinus with 0.1 ml of reconstituted LT vaccine of the homologous strain. At the same age, similar lots of birds from each vaccinated group were revaccinated by the same method used for initial application with a vaccine having a titer of $10^{6.2}$ EID_{50} per ml.

The sinus reactions in the challenged birds were read on the 4th day. The results given in table 2 show a high rate of susceptibility to reinfection when the birds were vaccinated by the drop or brush method irrespective of the age of vaccination. One observation to be noted was that those vaccinated birds that did react to the challenge virus exhibited less swelling, and the nasal discharge cleared up more rapidly than in the nonvaccinated controls. Although those birds vaccinated at 8 weeks appeared to be more resistant than those vaccinated at earlier ages, it should be noted that this age group had only an 8 week interval between vaccination and challenge. If this interval had been extended 7 more weeks, it is questionable that the result would have been any more favorable than in those birds vaccinated at one week of age.

The "take" readings on the revaccinated birds were also read on the 4th day (table 3). The "takes" were read critically starting with the group vaccinated initially at one week of age. In retrospect, because of the subjective readings, it is doubtful that seven birds recorded as having weak "takes" in the revaccinated group are significant. The virulence of the vaccine used was confirmed by strong "take" readings in 8/10 and 9/10 of the birds vaccinated initially by the drop and brush methods, respectively.

Three weeks postrevaccination, all birds were challenged by sinus inoculation with the homologous virus using 0.1 ml of a 1:2 dilution of vaccine having a titer of $10^{6.5}$ EID_{50} per ml. The sinus reactions were read 4 days postchallenge (table 4). The groups vaccinated initially at one and 4 weeks of age clearly showed that revaccination with the brush

TABLE II

Comparison of immunity resulting from drop vs brush methods of LT vaccine application at one, 4 and 8 weeks of age

Age vac.	Age challenged	Interval postvac.	Challenge Reaction: Vaccination Method: Drop	Challenge Reaction: Vaccination Method: Brush	Contact** Controls	Non-vac. Controls
1 week	16 weeks	15 weeks	10/10*	10/10	4/4	
4 weeks	16 weeks	12 weeks	9/10	8/10	5/5	
8 weeks	16 weeks	8 weeks	8/15	5/14	4/4	
Not Vac.	16 weeks					10/10

* No. susceptible/No. challenged
** Placed with group 10 days postvaccination

TABLE III

LT "take" reading after revaccination by method corresponding to that used for initial vaccination

Age initial vac.	Age revac.	Revac. interval	"Take" Reading on Revac.: Drop S	W	N	Brush S	W	N	Controls-Initial Vac.: Drop S	W	N	Brush S	W	N
1 week	16 weeks	15 weeks	0	2	10	0	5	5						
4 weeks	16 weeks	12 weeks	0	0	14	0	0	13						
8 weeks	16 weeks	8 weeks	0	0	10	0	0	10						
16 weeks	none	none							8	1	1	9	1	0

S = strong; W = weak; N = negative

method gave a better revaccination response than the drop method. There was no detectable difference in the two methods in the 8-week-old group. Those birds vaccinated initially at 16 weeks were completely protected by either method of application.

A second revaccination trial was conducted in birds from a commercial flock that had been vaccinated between 10 and 18 days of age with a triple virus vaccine* by the vent drop method. Seventeen weeks postvaccination 40 birds were obtained and divided into four groups. Five birds were bled from each group for pre-revaccination SN tests. Groups of 10 birds each were revaccinated by the vent drop, vent brush and eye drop methods and 10 were left as controls. The revaccinated birds were housed together while the controls were held separately. Observations on the reaction to the vaccine were made on the 4th day. None of the birds showed evidence of reaction to the vaccine whether administered by vent drop, vent brush or eye drop.

Four weeks post-revaccination, the same birds that were bled prior to revaccination were bled again. All birds, plus nine nonvaccinated birds from another experiment, were then challenged by infraorbital sinus inoculation of 0.1 ml of homologous virus suspension having a titer of $10^{5.5}$ EID_{50} per ml. The sinus reactions were read 5 days postinoculation. A summary of the post-revaccination and postchallenge reactions are shown in table 5. This trial confirmed the previous observation that revaccination in the vent does not elicit a noticeable reaction, but in view of the fact that nonrevaccinated controls showed a high level of protection 17 weeks after vaccination, the comparative revaccination methods did not give any conclusive results.

The SN results of the pre- and post-revaccination serum samples (table 6) revealed no significant increase in antibody titer in the birds vaccinated by the vent methods, but three of the five revaccinated by eye drop did show an increased titer and all birds had significant titers.

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* TRIPLVAX™, produced by American Scientific Laboratories, Inc., Madison, Wisconsin

TABLE IV

Results of infraorbital sinus challenge for LT 3 weeks post-revaccination

Age initial vac.	Age revac.	Interval post-revac.	Challenge Reaction		
			Vaccination Method		Nonvaccinated
			Vent Drop	Vent Brush	Controls
1 week	16 weeks	3 weeks	8/10*	0/10	
4 weeks	16 weeks	3 weeks	8/14	1/12	
8 weeks	16 weeks	3 weeks	1/10	1/10	
16 weeks	None	3 weeks postvac.	0/10	0/10	
None	None	None			12/12

* No. susceptible/No. challenged

TABLE V

Summary of revaccination response and challenge reaction in birds revaccinated by 3 different methods

Revaccination method	Vaccine Reaction Number revaccinated	Challenge Reaction Number challanged
Vent Drop	0/10	2/9
Vent Brush	0/10	1/10
Eye Drop	0/10	0/10
Nonrevaccinated Controls		1/8
Nonvaccinated Controls		8/9

TABLE VI

SN response to LT revaccination 17 weeks after initial vaccination of birds when 10 to 18 days of age with triple vaccine

Revaccination method	Bird no.	Log_{10} SN Titer Prerevac.	Log_{10} SN Titer 4 weeks postrevac.
Vent Drop	11	1.0	0.8
	13	1.0	1.1
	14	0.9	0.8
	16	0.7	0.8
Vent Brush	33	0.9	1.1
	35	0.7	1.1
	36	0.7	0.4
	37	1.1	2.1
	39	1.3	1.1
Eye Drop	21	1.0	>3.0
	22	0.3	2.0
	24	0.9	1.4
	25	1.1	>3.0
	26	0.9	1.2
Not Revaccinated	2	1.0	1.1
	3	0.9	0.2
	4	1.0	1.2
	5	0.9	0.8
Nonvaccinated Controls	41		0.9
	42		1.0
	43		0.9
	44		1.0

In a third trial on revaccination, 48 7-week-old White Leghorn cockerels were vaccinated initially by the vent drop method with a vaccine that had a titer of $10^{6.2}$ EID_{50} per ml. The "take" readings 4 days post-vaccination were recorded as 15 strong, 17 weak and 16 negative.

A similar number of cockerels from the same group were held in another house without vaccination. Eight weeks later (15 weeks of age), birds from the vaccinated group were revaccinated in groups of 10 by each of four methods using the same type vaccine as was used initially: vent drop, vent brush, nostril drop, eye drop. These were maintained in one colony house with five nonrevaccinated birds serving as contact controls. An equal number of birds was vaccinated initially by each of the four methods. These were held in another house along with contact controls. Five birds were bled from each of the revaccinated groups to determine the SN titer.

Four days postvaccination, all birds were checked for a "take" response. Reddening and swelling of the vent were the criteria of a response in birds vaccinated by the vent drop or vent brush methods; nasal discharge or lacrimation if vaccinated by the nostril or eye drop methods. The revaccination reaction was absent with each of the four methods of application with the exception of one bird which showed some lacrimation in one eye (table 7). The vaccine used was

TABLE VII

LT revaccination response and challenge reaction. Comparison of 4 methods of vaccine administration

Revaccination method	Revac. "take" reaction 4 days post-revac.	Sinus reaction to challenge 5 weeks post-revaccination
Vent Drop	0/10	6/9*
Vent Brush	0/10	5/10
Nostril Drop	0/10	4/10
Eye Drop	1/10	1/10
Contact Controls	0/5	1/5
Nonvac. Controls		9/9

* No. susceptible/No. challenged

potent as seen by the reactions shown in the birds vaccinated initially (table 8). Birds vaccinated by the nostril drop method showed very little reaction with only one of 10 exhibiting nasal discharge. With direct application of the vaccine to the eye, only five of 10 birds manifested a reaction. During the postvaccination period, the contact controls in both houses failed to show clinical signs of laryngotracheitis.

TABLE VIII

LT initial vaccination response and challenge reaction. Comparison of 4 methods of vaccine administration

Vaccination method	Take reaction 4 days postvac.	Sinus reaction to challenge 5 weeks postvac.
Vent Drop	9/10	1/9*
Vent Brush	10/10	0/10
Nostril Drop	1/10	1/10
Eye Drop	5/10	0/9
Contact Controls	0/4	1/4
Nonvac. Controls		9/9

* No. susceptible/No. challenged.

Five weeks postrevaccination, the same five birds from each group were again bled for the SN test. Five birds from each of the groups vaccinated initially were also bled. At the time of bleeding, all birds plus nine nonvaccinated controls were challenged by infraorbital sinus inoculation of 0.1 ml of reconstituted vaccine with a titer of $10^{5.5}$ EID_{50} per ml. Four days later, all birds were examined for evidence of sinus reaction to the challenge virus. These results are also presented in tables 7 and 8. In comparing the protection afforded the various groups, it can be seen that the birds vaccinated initially were better protected by vent drop, vent brush and nostril drop methods of administration than in those groups that were revaccinated by the same routes. As has been observed previously, the sinus reaction in vaccinated bids was less pronounced and of shorter duration than in the nonvaccinated controls. There was good protection where the eye drop method was used, whether applied initially or as a revaccination. Seven of the nine contact controls in the two houses showed evidence of having had sufficient exposure to the virus to develop immunity. This is significant when compared to the nonvaccinated isolated controls which showed nine of nine infected upon challenge.

The SN results on the blood samples drawn from the various vaccinated groups in trial 3 are shown in tables 9 and 10, along with the resulting sinus reaction to the challenge virus. In evaluating the revaccination response by the SN

TABLE IX

SN response and challenge protection to LT revaccination. Comparison of 4 methods of vaccine administration

Revaccination method	Bird no.	Log_{10} prerevac.	SN titer postrevac.	Sinus reaction to challenge
Vent Drop	7194	0.0	1.1	+
	7159	0.9	2.4	—
	7154	0.6	<0.9	—
	7151	1.6	1.8	+
	7152	0.4	<0.9	+
Vent Brush	7160		2.6	+
	7155	0.6	1.7	—
	7186		>2.9	—
	7167	0.0	2.2	+
	7183	0.7	2.0	+
Nostril Drop	7189	0.1	<0.9	—
	7164	1.0	0.9	+
	7188	0.0	1.4	—
	7162	0.9	2.4	+
	7184	0.6	<1.9	—
Eye Drop	7181	0.1	3.2	—
	8498	0.7	>3.1	—
	7197	0.1	3.9	—
	7178	1.3	3.1	—
	7179		2.2	—

results, the vent drop and nostril drop methods were relatively poor. While the vent brush method stimulated a better serologic response, the resulting protection to sinus challenge appeared to be no better than when the vent drop or nostril drop methods were used. The eye drop method looked most promising as a revaccination technique both from the standpoint of serologic response and protection against sinus challenge. In the birds vaccinated initially by the four methods, the results are comparable whether measured by serologic response or sinus reaction to challenge, with possibly some slight advantage in the eye drop method.

Of particular interest in this trial was the lack of correlation between the SN results and the resistance to sinus challenge in birds 5 weeks after vaccination. In prior studies[2] a log_{10} titer of 1.0 or greater indicated previous experience with LT virus. In reviewing the present results, it can be

TABLE X

SN response and challenge protection to LT initial vaccination. Comparison of 4 methods of vaccine administration

Vaccination method	Bird no.	Log_{10} prevac.	SN titer postvac.	Sinus reaction to challenge
Vent Drop	8234		1.8	
	8324		2.4	+
	7509		2.6	—
	7526		1.6	—
	8356		<0.1	—
Vent Brush	8329	0.1	1.1	—
	7590	0.0	2.6	—
	8325	0.0	1.1	—
	7506	0.0	3.1	—
	7598	0.0		
Nostril Drop	8301		0.6	—
	8352		<0.3	+
	7547		2.8	—
	8499		0.4	—
	7543		1.9	—
Eye Drop	8217		3.1	—
	7482		3.0	—
	8334		1.3	—
	8337		2.9	—
	8368		1.1	—
Nonvaccinated Controls	8178	0.7		+
	7584	0.9		+
	8203	0.1		+
	8393	<0.3		+
	7585	<0.9		+

seen that the SN results are of very little value in predicting a bird's resistance to virus challenge.

DISCUSSION

The results of these trials point out the fallacy of making claims for lifelong immunity following vaccination for LT. One might be misled to this assumption from failure to see a "take" reaction in the vent upon revaccination. However, the ability to infect the sinus proves reinfection is possible regardless of previous vaccination, and undoubtedly, helps to explain some so-called field "breaks." These data may be criticized for failure to use the trachea, the site of the natural infection, as the route of challenge. The sinus was chosen be-

cause it permits greater objectivity in the visual evaluation of the results and is reproducible experimentally. It is doubtful whether the use of tracheal challenge would have changed the results materially.

The need for revaccination in certain circumstances is readily apparent. Failure to accomplish a boost in immunity by the vent drop method in three trials would rule out this method. The vent brush method induced a serologic booster response in two of three trials, but the failure to show a direct correlation between serologic titer and challenge protection (table 9) points out the deficiency of the brush method. The nostril drop method gave an erratic response on the initial as well as the revaccination trials, and therefore, does not offer the most desirable procedure.

The eye drop method seems to be the best revaccination procedure in view of the serologic response and resistance to subsequent challenge. This method is simple and one with which the poultrymen are well acquainted from previous experience with the ocular administration of Newcastle disease and infectious bronchitis vaccines. The eye drop method for revaccination appears to be inherently safe, since, even in birds vaccinated initially by this route (table 8), clinical signs were only mild, local and transient in five of the 10 birds. Before adopting the ocular route as a method for revaccination for LT, however, it would be necessary to test it further under field conditions. While the revaccination reaction in these trials was of no consequence, it should be determined if the same would be true when used in the field where other complicating infections exist.

SUMMARY

1. Birds vaccinated against laryngotracheitis in the vent by the brush or drop methods at one, 4 and 8 weeks of age, respectively, showed a high degree of susceptibility when challenged at 16 weeks of age by the infraorbital sinus route.

2. Revaccination by the vent route (either by drop or by brush method) failed to elicit a "take" reaction and failed to induce the desired booster response for increased protection.

3. Revaccination by dropping vaccine in the eye showed

the greatest promise for stimulating increased resistance as revealed by the serologic response and protection upon challenge.

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ABSTRACTS OF PAPERS PRESENTED AT THE 9th ANNUAL WESTERN POULTRY DISEASE CONFERENCE, FEBRUARY 1, 1960, DAVIS CALIFORNIA

Some papers presented at the conference are printed in completed form elsewhere in this issue. Other papers are represented by the abstracts below.

PREVENTING EGG-BORNE MYCOPLASMOSIS IN TURKEYS

H. E. Adler
University of California, School of Veterinary Medicine, Davis, California

No abstract submitted.

WE CAN TREAT PET BIRDS

R. W. Wichmann
University of California, School of Veterinary Medicine, Davis, California

No abstract submitted.

LARYNGOTRACHEITIS VACCINATION "BREAKS"

L. G. Raggi
University of California, College of Veterinary Medicine, Davis, California

No abstract submitted.

INFECTIOUS CORYZA AND *HEMOPHILUS*

W. D. Urban
Ontario, California

No abstract submitted.

LANDMARKS IN AVIAN ENCEPHALOMYELITIS

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Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y.

Since the first recognition of avian encephalomyelitis (AE) as a viral entity in 1932 only about 28 reports have been published. The prevalence of the paretic form emphasized the need for histologic differentiation from clinically similar, nutritional and infectious disorders. The diagnostic lesions were found to be roundish, circumscribed lymphoid follicles in the pancreas, lymphoid infiltrates in the proventricular muscularis, and glial and perivascular foci in the brain, especially medulla, cerebellum and diencephalic nucleus ovoidalis, accompanied by axonal reaction of the large neurons. The association of cataracts with the disease in recovered birds was an interesting finding. Chicks, hatched from inoculated eggs, developed the disease and the virus was recovered from the unabsorbed egg yolk of 2-day-old chicks, as in the pullorum cycle.

The observation that the production of AE infected chicks was preceded by a temporary slump in egg production of their dams made vaccination of prospective breeding stock with a crude chick brain vaccine feasible. Chicken embryos infected via the yolk sac and eye routes did not die but became paralyzed owing to extensive muscle and brain damage. Eggs of high susceptibility were found to come from susceptible dams, those of low susceptibility from immune dams. By the egg test most flocks showed evidence of prior infection; susceptibility proved to be the exception—a complete reversal of the former epizootiologic concept. AE virus was recently grown in chick kidney tissue cultures in which the virus grew to a high titer, caused a characteristic cytopathic effect preceded by the formation of basophilic intranuclear inclusion bodies, and could be used for an *in vitro* serum neutralization test. Survival of the virus at 37 C for 12 days testified to its tenacity.

ISOLATION OF AN UNIDENTIFIED MYXOVIRUS FROM CHICKENS WITH A RESPIRATORY DISEASE

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During investigation of an outbreak of infectious laryngotracheitis (LT) with an unusually high mortality, an unidentified filterable agent was isolated in chicken embryonating eggs inoculated with the tracheal exudates. The LT virus was removed from the mixture by treating infected allantoic and amniotic fluids (aa fluids) with hyperimmune LT chicken serum.

The agent, which hemagglutinates chicken erythrocytes, was not neutralized by anti-Newcastle disease chicken serum, and recovered chickens were susceptible to a challenge dose of the GB strain of NDV. On further study, the agent was found to be highly stable, having a particle size similar to the NDV. It produces a sporadic mortality in chicken developing embryos, and the aa fluids of the latter consistently result in an infectivity titer of 1×10^8 per 0.2 ml of inoculum. The agent is cytopathic for HeLa and pig kidney cell tissue cultures.

Intratracheal inoculation into susceptible chickens produces a mild respiratory distress characterized by moist rales between the 4th and 6th days. No mortality was observed following intratracheal or intramuscular inoculation into chickens 4 to 7 weeks old. Serums from chickens recovered from infection specificially inhibited hemaggluti-

nation of chicken red blood cells with its homologous virus, but not NDV; thus, it can be readily differentiated from the more frequently encountered agent. No cross hemagglutination inhibition reactions in serums from recovered birds were observed between this agent and NDV. The agent was not neutralized by antiserums of the infectious bronchitis, fowl plague, influenza A, B, and C, mumps, quail bronchitis, and parainfluenza types 1, 2, and 3 viruses and mycoplasmas.

A survey was made of chicken serums obtained from 169 flocks with respiratory infections that were submitted to the poultry pathology diagnostic laboratories in Los Angeles and Stanislaus counties. Antibodies to the new agent were detected in the serums of three diseased flocks. While this agent does not appear to be widespread at the present time, it may be capable of or instrumental in triggering or aggravating otherwise silent infections into full-scale, serious respiratory disease outbreaks.

COCCIDIOSIS VACCINATION

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To test the efficiency of coccidiosis vaccination for replacement pullets, 10,000 White Rock pullets on 4 farms were given coccidiosis vaccine (Dorn and Mitchell) in the drinking water at 4 days of age. The material contained sporulated oocysts of *E. tenella, E. necatrix, E. maxima, E. hagani* and *E. acervulina.* All chicks were fed 20 percent protein starter containing .04 percent Glycamide for the first 6 weeks. No unvaccinated controls were maintained.

When the birds were 27 weeks old, cull pullets or cull and surplus males were selected for challenge. Six-week-old battery reared broiler-type chicks susceptible to coccidiosis were inoculated simultaneously to assess the virulence of each of the coccidial cultures used and are called controls.

Attemped challenge with *E. acervulina* and *E. hagani* did not produce infection and lesions typical for the species in the controls. Results were inconclusive and are not listed.

Eighty-seven principals and 10 controls each received 100,000 *E. tenella* oocysts; 30 other principals and 5 controls received 40,000 *E. necatrix* oocysts; 30 principals and 5 controls received *E. maxima* oocysts; while an additional 30 principals and 5 controls received *E. brunetti* oocysts as challenge.

With these 4 species infection occurred and lesions were produced in all of the controls; whereas few lesions were produced by challenge in the principals. Increased resistance to *E. brunetti* was demonstrated in the principals even though *E. brunetti* was not included in the vaccine. It must therefore be assumed that at least some of the immunity demonstrated in the vaccines was a result of low-level natural exposure from the environment. It was not possible to assess effect of the environmental exposure because nonvaccinated controls were not kept on each premise.

Prior to and after this trial coccidiosis had been a problem on the farms during the growing period, while these broods were raised without problems from coccidiosis and without using a coccidiostat after the 6th week. These results indicate a possible advantage, based on cost, of planned exposure as contrasted to use of a coccidiostat. The cost of vaccination vs. continuous feeding of a coccidiostat was discussed. The advantage of one over the other depends on the cost of the coccidiostat, the purpose for which the birds are raised, feeding and management systems used, and degree of contamination of the environment with coccidia.

DRUGS AND CARCASS QUALITY

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The economic advantages or limitations of drugs are evaluated in terms of their effect on field performance, carcass condemnation, processing plant costs, and carcass quality. Field performance—namely, rate of gain—is not as critical an indication of flock health or drug efficiency as the condemnation rate.

While diseases such as CRD affect field performance, medication frequently cannot reverse disease processes and thus carcass condemnation remains high despite fairly satisfactory field performance. In order to be most effective, medication must be applied during an outbreak early enough and in adequate amounts to permit recovery of the birds and healing of lesions. In order to justify expense, drugs must reduce condemnations by at least one-half or more and otherwise improve field performance. This is seldom accomplished. A high number of birds with disease, lesions, or of poor quality add processing costs because it is necessary to slow down the line and have additional personnel. Use of improved and more effective vaccination programs, based on accurate diagnosis and knowledge of area or farm problems, correct and discriminate medication, and good preventive management are suggested as most efficient ways to reduce condemnations, improve performance and carcass quality. The economic feasibility of any program must be based on the cost compared to the changes attained in field performance, carcass condemnation rates, processing plant costs, and ultimate carcass quality.

ERYTHROMYCIN THERAPY IN POULTRY DISEASES

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In poultry feeding, tissue diffusion of erythromycin was evident at 20 to 200 gm/ton levels. This may contribute to its therapeutic effectiveness in poultry diseases. The injection of erythromycin ethyl succinate, intramuscularly or subcutaneously, at levels of 5 mg/lb body weight produced blood levels of therapeutic significance within one hour. This is measured by the anti-PPLO activity of the blood after the erythromycin injection and after the serum is diluted in twofold steps. The experimental injection of erythromycin at the rate of 15 mg/lb body weight into layers resulted in levels in the egg inhibitory for from 48 to 72 hours to most micro-organisms sensitive to this antibotic. The LD_{50} of injectable erythromycin is approximately 110 to 140 mg/lb body weight of bird (chicken or turkey). The injection of this antibiotic in layers (properly handled during the injection) had no deleterious effects on production. Controlled experiments have shown that a dosage of 50 mg/adult bird will show a significant suppression of PPLO organisms in ova transmission as shown in culled and pipped eggs. Many workers have shown erythromycin to be effective in turkey sinusitis when the antibiotic is injected into the sinus. Recent work has shown that under experimental conditions erythromycin was effective in suppressing PPLO organisms in eggs dipped in a 400 ppm concentration of the antibiotic for a definite period of time.

Experiments using erythromycin dust via a nebulizer tend to show suppression of CRD organisms. However, this is very difficult to evaluate in relation to later disease as the birds grow and become exposed to various concentrations of infective organisms in the field.

Low level feeding (50 gm/ton) has shown controversial results in reducing the incidence of disease outbreaks but on many occasions has enhanced the growth rate. Clinically, CRD often responds to levels of erythromycin in feed (100 to 300 gm/ton) and in water (75 ppm). Assayable erythromycin blood levels, by our technics, are not detectable below the maximum feed and water levels mentioned. In experiments conducted by Miner and Smith (Utah) and Olson (West Virginia), no significant therapeutic effects resulted after feeding erythromycin to turkeys infected with staphylococcosis, or to chickens with infectious synovitis of virus etiology. Infectious synovitis in chickens did not respond to injectable erythromycin.

TREATMENT OF CHRONIC RESPIRATORY DISEASE WITH TYLOSIN

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The efficacy of tylosin, a new antibiotic, against experimental pleuropneumonia-like organism (PPLO) infections and naturally occurring chronic respiratory disease (CRD) of chickens was studied. Experimental PPLO infections and tylosin treatments were evaluated by the following criteria; weight gain, serology, gross and histological lesions, PPLO and bacterial isolations, and isolation and serological tests for the presence of respiratory viruses. Field cases of chronic respiratory disease were diagnosed and tylosin treatment evaluated by the following criteria; weight gain, feed conversion, mortality, lesions and condemnation at slaughter, serology, PPLO and bacterial isolations, and isolation and serological tests for the presence of respiratory viruses.

Summaries of experimental PPLO infections treated with tylosin showed that 5 mg per pound of body weight intramuscularly or intraperitoneally, 2.5 gms per gallon of drinking water for 5 days or 50 mg of dust (inhalation) per square foot of floor space, three times at 24-hour intervals is highly effective. Close correlation was seen between all measurement criteria. Summaries of field cases of chronic respiratory disease treated with tylosin showed that 10 mg per pound of body weight intramuscularly or 2 gms per gallon of drinking water for 5 days is an effective treatment. Close correlation was observed between the various measurement criteria.

KILLED AND LIVE VACCINES PLUS MANAGEMENT

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Most poultry ranches built in Southern California during the past few years were designed so that the brooding and growing areas are in close proximity. Most ranches brood year around on a schedule as close as 3 weeks apart. Many independent ranches are very close neighbors and the disease problems of one ranch have become the mutual problems of their neighbors. Nearly all birds are brooded, grown and spend their laying life on wire in cage type operations.

Once the so called "respiratory complex" which is common in this area is established on a ranch it is there to stay as long as continuous brooding is practiced and no drastic management changes are made. The respiratory disease complex of this area is made of mixed viral and bacterial infections. Often it is a mixture of Newcastle disease, infectious bronchitis, infectious coryza, pasteurellosis, and PPLO infection.

Spacing the broods at least 7 weeks apart and separating birds of different ages from each other and from the brooders has brought great

improvement to many ranches. Designing a vaccination program to get a high degree of immunity to the viral diseases by the time the birds are 7 weeks old with a minimum of stress has been helpful. Boosters are given for bronchitis and Newcastle disease before the pullets are mature.

The brooders should be 200 feet or more from all birds. Birds of different ages should be isolated 200 feet from each other but not over 100 feet from the layers. This will probably vary according to wind and weather conditions. By keeping the growers well isolated from each other we help prevent the build up of virulent pathogens. By exposing the growers to the more or less immune carriers of the laying flock that are not acutely infected, we effect a gradual exposure to the diseases present on the ranch before the birds come into production. This applies primarily to the bacterial diseases of the flock.

During recent months we have been preparing and field testing live and killed autogenous Hemophilus vaccines. The live vaccines have been administered when possible 2 weeks or more prior to the usual field exposure time. The killed vaccines are used on started pullets prior to shipping them to coryza contaminated ranches. The mortality and number of retarded replacement pullets has definitely been decreased. There is evidence of a great reduction in the CRD and Pasteurella problems following the immunity produced by these Hemophilus vaccines.

ANTIBIOTIC POTENTIATION IN POULTRY DISEASE CONTROL

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To date, four different methods have been used to potentiate the tetracycline antibiotics. These are: (1) use of terephthalic acid or its salts—these may function through slowing down the renal excretion rate of the antibiotics; (2) addition of acids or acid salts such as phosphates, citric acid, etc.—these apparently tie up calcium and increase absorption of antibiotics; (3) a temporary reduction in dietary calcium—this permits a greater quantity of antibiotic to be absorbed from the intestinal tract; and (4) use of dietary calcium sources such as calcium sulfate and calcium gluconate—these do not depress antibiotic absorption to the same extent as calcium carbonate and dicalcium phosphate. A number of studies have clearly demonstrated that the increase in blood serum levels induced by potentiation procedures results in a corresponding increase in therapeutic effectiveness of the antibiotics. The latter two methods appear to have the greatest applicability at the present time.

In broiler birds, lower dietary calcium for up to 5 days, or supplying dietary calcium as calcium sulfate, have proved under laboratory conditions to be relatively safe methods of potentiating antibiotics, although it has been demonstrated that a prolonged reduction in dietary calcium will produce a rachitic condition and that use of high levels of calcium sulfate may cause a wet droppings problem. Limited laboratory studies with laying hens have shown that a sharp drop in dietary calcium for birds in excess of 3 days results in depressed egg production and eggs with poor shell quality.

The effect of potentiating oxytetracycline on two diseases which involved the respiratory tract of chicks has been investigated. Data show that a temporary reduction of dietary calcium during antibiotic therapy in an experimental CRD infection markedly increased saleable birds as compared to treatments where the same level of antibiotic was administered in a normal diet. Similarly, the response of artificially induced fowl cholera to 100 ppm antibiotic in the drinking water when

birds were receiving a low calcium diet was just as great as the response obtained with 200 ppm antibiotic given in water concurrently with a normal calcium diet.

EXPERIENCE WITH FOWL POX VACCINATION

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Fowl pox has occurred commonly in breeder turkeys in California that have been vaccinated for this disease. A single vaccination just prior to maturity has not always given satisfactory results but seems to give better protection than an early and a late vaccination. Stick method vaccination on the wattle was better than on the thigh or the wing web. Application of the vaccine to the wattle may have the following advantages: (1) The "takes" can be observed on any number of turkeys without individual handling. (2) The birds cannot pick at their own "takes". (3) If the flock is to be slaughtered soon after vaccination for any reason, the location of the "takes" on the wattle will not affect the market value of the birds. (4) The location of "takes" on the wattle may give better protection to the head, the usual focal point of natural infection.

Extremely potent fowl pox vaccines probably should not be used on either chickens or turkeys because a dangerous infection is brought onto the premises. In chickens secondary pox lesions, on the head, may be produced and serious post- vaccination reactions may occur.

Trichomoniasis of the upper digestive tract occurred regularly in one area soon after vaccination of chickens for pox. A 1:2000 solution of copper sulphate in place of all drinking water was an effective prophylactic and therapeutic agent. The disease could be prevented if treatment was started about 10 days after vaccination and continued 3 or 4 days.

A serious skin disease in both unvaccinated and recently vaccinated chickens was found to be caused by a combination of fowl pox virus and a staphylococcus. Broad areas of the skin were involved, with extensive edema, necrosis, sloughing and loss of feathers. The condition could be reproduced by inoculating the skin with the pox virus and rubbing the staphylococcus culture over the pox lesions about ten days later. The combined infections spread widely over the body.

CASE REPORT—AN OUTBREAK OF ORNITHOSIS IN NEW MEXICO[1]

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Received 18 March 1960

ORNITHOSIS virus has been isolated from birds in many states as well as in foreign countries. Recent monographs edited by Beaudette[1,2] make a review at this time unnecessary. It has been well established that the turkey is a susceptible host for the agent of ornithosis.[4] The purpose of this paper is to describe the first case of ornithosis occurring in turkeys in New Mexico. The diagnosis was made on the basis of clinical, pathological, and virological findings.

Six 9-week-old Broad Breasted turkeys, all alive, were submitted to the laboratory in September, 1958. The owner reported that of a total of 2,200 turkeys in the group, 130 to 150 had died during the week preceding the submission of birds for diagnosis. There was a decrease in feed and water consumption. Clinically, they exhibited a sulfur-colored diarrhea and cyanosis of the heads. The birds were dull and listless.

The gross post-mortem lesions consisted of a pericarditis with a caseo-fibrinous exudate. Similar exudates over the surface of the liver and serous membranes of the abdominal and thoracic cavities were also observed. The liver and spleen were enlarged. These lesions were present in various degrees in all birds submitted.

Bacterial cultures of spleen, heart blood, and liver were negative. Pooled samples of spleen, kidney, and liver exudate were treated as described by Meyer and Eddie[5] and Davis.[3]

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[1] Journal Series No. 145, New Mexico Agricultural Experiment Station, University Park, New Mexico.

The pooled samples were then inoculated in several series of 9-day-old chicken embryos via the allantcic cavity. Streptomycin was used at the rate of 25 mg per ml to control contamination. Embryo mortality began on the third day and occurred as follows: D_3, D_3, D_4, D_4, D_7, and A_8. All eggs in the second and third passages died between the 3rd and the 8th day after inoculation. The embryos became sluggish before dying. Lesions exhibited after death were generalized hemorrhage and a clouding and thickening of the embryonic membranes. Impression smears of the chorioallantoic membranes stained by the method of Giemsa revealed many LCL inclusion bodies upon microscopic examination.

Chlortetracycline medication at 200 grams per ton of an all-mash ration was started immediately after the post mortem examination was completed. The owner reported that within 5 days deaths dropped to one to two per day, and the flock exhibited considerable improvement. There was a cessation of deaths after a 3-week period. Some 300 turkeys died during the outbreak. The chlortetracycline at 200 grams per ton was fed for a month before it was discontinued. There were no reported illnesses in humans coming in contact with the infected turkeys.

SUMMARY

The first outbreak of ornithosis in turkeys occurring under natural conditions in New Mexico is reported. LCL bodies were demonstrated from impression smears of membranes of chick embryos dead from inoculation with infectious material.

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A VARIANT TYPE OF INFECTIOUS BRONCHITIS VIRUS IN A COMMERCIAL VACCINE*

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INFECTIOUS bronchitis (IB) is a very common virus disease of chickens. The virus attacks the respiratory system and, in the majority of cases, reduces egg production and impairs both the internal and external egg quality of susceptible layers. In the United States, IB is one of the poultry industry's most serious economic problems because in most instances, it affects production and quality of eggs; it may kill chicks; it stunts growing birds; in conjunction with *Mycoplasma gallisepticum* (PPLO), it causes a severe, respiratory disease complex.

Previous immunity studies[9] of seven commercially available IB vaccines and the Massachusetts strain of IB virus indicated similarities in their immunogenicity. The commercial IB vaccines and the IB virus isolates from California were antigenically similar. Differences in the antigenic make-up of IB viruses were suspected by Hofstad.[6]

Jungherr *et al.*[8] recently reported a new antigenic type (Connecticut type) of IB virus. This paper reports further study of the serological and cross-immune response of birds inoculated with the Connecticut type in a commercially available infectious bronchitis vaccine.

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* Presented at the XI World's Poultry Congress, September 21-28, 1958, Mexico City, Mexico.

MATERIALS AND METHODS

Vaccine. The IB vaccine, obtained in lyophilized form directly from the manufacturer in May, 1957, was immediately reconstituted in the diluent furnished by the manufacturer and stored in a refrigerator at 2 C. Examination for bacterial contaminants, Newcastle disease and infectious laryngotracheitis viruses, gave negative results.

Experimental birds. Four groups of birds were used in this experiment, as outlined in table 1. Group 1 consisted of 2-month-old White Leghorn males received as day-old chicks from the flock of the Department of Poultry Husbandry, University of California, Berkeley. This flock had not been vaccinated against any disease. It is known to be susceptible to IB and Newcastle disease and free of *Mycoplasma gallisepticum* agglutinins[1] for the last 5 years. The birds we used were isolated until the experiment began.

Group 2, 1½ year-old layers obtained from the same source at the age of 1 year, were also isolated until the experiment began. The birds' average egg production and egg quality were established during the month preceding these studies and also following vaccination. One week before the beginning of the experiment, 10 birds from this group were inoculated intratracheally with at least 10,000 chicken $i.d._{50}$ of IB virus, Massachusetts strain, and found to be susceptible.

Group 3 comprised 43 6-month-old unsexed White Leghorns from the same source as groups 1 and 2. They were vaccinated with 10,000 $i.d._{50}$ of the Massachusetts type of IB virus at the age of 2 months and kept isolated until challenged.

The day the experiment began, all birds were bled and the sera were tested individually for serum neutralizing antibodies against the egg-adapted IB virus. The ELD_{50} of this virus was in the range of 10^{-8}. This virus had been neutralized consistently by the immune sera of birds previously inoculated with the IB virus strains isolated in California.

The birds were kept isolated, numbered, and vaccinated intranasally with one drop of the reconstituted vaccine. All birds in group 1 were examined daily for respiratory signs. Seven weeks after the vaccination of groups 1 and 2, and 4

months after the vaccination of group 3, all birds were rebled, and the sera were individually tested for neutralizing antibodies of IB virus.[3,6]

On the same day, group 1 was divided into subgroups A and B, group 2 into subgroups C and D, and group 3 into subgroups E and F. The number of birds in each subgroup varied between 19 and 23. Each subgroup was placed in a new metal cage in the middle of an uncultivated field. Each subgroup was about one-half mile from the other and so located as to minimize the spread of infection by the prevailing wind.

Subgroups A, C, and F were challenged intratracheally with the undiluted reconstituted vaccine, the same vaccine used to vaccinate the birds intranasally. The challenge dose of virus was at least 10,000 embryo $i.d._{50}$ administered with a 2 ml syringe with a slightly bent 20 gauge needle. Subgroups B, D, and E were challenged intratracheally with at least 10,000 chicken $i.d._{50}$ of the Massachusetts strain of IB virus.

Control group 4 was 43 7-month-old layers acquired from a nearby ranch one month before challenge. No vaccination was practiced by the owner, and three birds from the group were found susceptible to an intratracheal challenge of at least 10,000 chicken $i.d._{50}$ of the Massachusetts strain. During this month their average egg production and quality were determined. This group was separated into subgroups G and H, of 20 layers each. Subgroup G was inoculated intratracheally with the undiluted commercial vaccine, and subgroup H with 10,000 chicken $i.d._{50}$ of the Massachusetts strain. The effect of both strains of virus on egg production and quality was recorded.

Following challenge, all birds were examined daily for 15 days for respiratory symptoms. Those birds that did not show respiratory signs were considered immune. Those that gurgled, sneezed, or coughed were considered susceptible.

RESULTS

The IB virus-neutralizing titers of the prevaccination sera varied between 1 and 10, which were considered nonspecific. Challenge results are summarized in table 1. Table

TABLE I

Response of birds to an infectious bronchitis vaccine. Groups A, B, C and D challenged 7 weeks after vaccination. Groups E and F challenged 4 months after vaccination.

Group	Sub-group	No. of birds	Age and sex	Vaccine virus	Morbidity %	Challenge virus	No. of birds showing respir. signs	Immunity to challenge %
1	A	19	2 mo. males	Connecticut	52.6	Connecticut	0	100.0
	B	20	2 mo. males	Connecticut	65.0	Massachusetts	15	25.0
2	C	19	18 mo. females	Connecticut	†	Connecticut	1	94.7
	D	20	18 mo. females	Connecticut	†	Massachusetts	12	40.0
3	E	23	6 mo. mixed	Massachusetts	100.0	Massachusetts	0	100.0
	F	20	6 mo. mixed	Massachusetts	100.0	Connecticut	4	80.0
4	G	20	7 mo. females	none	†	Connecticut	11 (55%)	—
	H	20	7 mo. females	none	†	Massachusetts	19 (95%)	—

† Birds not examined to avoid affecting egg production

2 summarizes the serological responses of the birds 7 weeks after vaccination. No differences were detected between pre-inoculation and post-inoculation egg production and qualities.

TABLE II

Serum neutralization titers* 7 weeks after vaccination but prior to challenge

	GROUP 1 (Young cockerels)		GROUP 2 † (Layers)	
	Titer	No. birds	Titer	No. birds
ASPECIFIC	10^0	31	10^0	6
	10^1	7	10^1	21
SPECIFIC			10^2	6
	10^3	1	10^3	2
			10^4	1

* Determined in chicken embryonating eggs by using the egg-adapted infectious bronchitis virus, DA 138.
† Three birds not bled.

DISCUSSION

Table 1 shows that the immune response of the birds to the commercial vaccine strain, as judged by the homologous challenge, was excellent in subgroups A and C. Subgroup A showed 100 percent immunity; subgroup C showed 94.7 percent immunity. The immune response of the birds in subgroups B and D was poor when challenged with the Massachusetts strain. Only 25 percent of the birds of subgroup B and 40 percent of the birds of subgroup D were found immune. That chickens vaccinated with an IB vaccine can develop immunity in 7 weeks, is clearly demonstrated in this experiment by their satisfactory response to a homologous challenge.

The relatively favorable immune response of subgroup F to challenge with the Connecticut type (80 percent immunity) is more apparent than real. Admitting that the 7-month-

old layers used in subgroup G could not be used, strictly speaking, as controls for subgroup F, they were, nevertheless, of a comparable age (only one month difference). They were susceptible to infectious bronchitis and inoculated intratracheally by the same method used for challenging the birds of subgroup F. Since only 11 of 20 birds (55 percent) of subgroup G showed respiratory signs following intratracheal inoculation, and since susceptibility to challenge of group F is based on the presence of respiratory symptoms following intratracheal inoculation, it is permissible to assume that the percentage of birds susceptible to challenge in group F (20 percent) would have been increased considerably had we the opportunity to compare the Massachusetts type of infectious bronchitis with one more pathogenic than the mild Connecticut type.

Table 2 indicates that 38 of the birds vaccinated at 2 months of age had non-significant titers varying between 1 and 10 7 weeks after vaccination; only one bird showed an IB virus-neutralizing titer of 10^3 at this time. The same table also shows that 27 of the vaccinated layers showed a non-significant titer varying between 1 and 10 7 weeks after vaccination. Six birds had a titer of 10^2, two birds had a titer of 10^3, and one bird had a titer of 10^4 IB virus-neutralizing doses. It has been demonstrated [2,3,4,7,10] that the sera of IB-susceptible birds will not show more than 10 virus-neutralizing doses.

There was a partial cross immunity between the Connecticut type and the Massachusetts type challenge virus. As a whole, the serological response was poor when tested against the egg-adapted strain of IB virus. A few birds, however, showed a specific rise in titer. The same birds, however, were susceptible to challenge. Noticeable discrepancies between the SN titers and the challenge results were previously reported.[10]

Assuming the strain used in the vaccine was the same as that reported by Jungherr, the present findings indicate this strain must have some antigenic components in common with the Massachusetts type.

The absence of detectable effects of both the Connecticut

and the Massachusetts strains of IB virus on egg production and egg quality (Haugh units and shell thickness) of susceptible layers is considered an exception to the usual experience. The results do not invalidate the importance of IB as far as its deleterious effects on egg quality and egg production but they emphasize the danger of assuming that IB in susceptible layers is always followed by a drop in egg quality and in production. Hill[5] and Zander and Rosenwald[11] observed no detectable drop in infected, susceptible layers.

Loss in egg production reported after outbreaks of IB in California flocks vaccinated with the above vaccine seem to confirm the experimental results reported herein.

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The author is indebted to Mr. G. G. Lee for conducting the serum neutralization tests and for his invaluable assistance in all phases of this work.

SUMMARY

Cross immunity in serological studies with a type of infectious bronchitis (IB) virus (Connecticut type) as found in the commercially available IB vaccine indicated that:

1. Only five of 20 2-month-old birds and eight of 20 18-month-old layers vaccinated with the Connecticut type of IB virus resisted an intratracheal challenge with the Massachusetts type IB virus, while 20 of 20 of the 2-month-old and 19 of 20 of the 18-month-old layers resisted a homologous challenge.

2. Sixteen of 20 6-month-old birds inoculated with the Massachusetts type of IB virus resisted an intratracheal challenge with the Connecticut type virus, while 23 of 23 birds of the same age and source resisted a homologous challenge with the Massachusetts type virus.

3. One of 39 serum samples from the birds vaccinated at 2 months of age, and nine of those vaccinated at 18 months showed specific IB virus neutralizing titers (10^2 or higher) immediately before challenge. The above ten birds, however, were susceptible to challenge. The total lack of correlation between the pre-challenge IB neutralizing titers and the ex-

pected behavior of the birds to challenge is surprising and cannot be explained satisfactorily at this time.

4. The results demonstrated the existence of a variant of IB virus with some antigenic component in common with the standard IB virus represented by the Massachusetts strain.

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RESEARCH NOTE—A RAPID MACROSCOPIC PLATE AGGLUTINATION TEST FOR NEWCASTLE DISEASE— A PRELIMINARY REPORT

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A RAPID and simple macroscopic test to detect antibodies against animal viruses would be of value in human and veterinary medicine. This paper (1) describes a plate agglutination test to detect antibodies for Newcastle disease (ND).

The antigen was prepared from centrifuged chorio-allantoic fluid of embryonating chicken eggs infected with the GB (Texas) strain[1] of ND virus. Eighty 9-day-old embryonating eggs were inoculated via the chorio-allantoic route with one million ELD_{50} of the GB (Texas) strain of ND virus. The chorio-allantoic fluid (360 ml), harvested at death of the embryos 18 hours or more after inoculation, was differentially centrifuged. Extreme care was taken in harvesting asceptically only the chorio-allantoic fluid. If yolk material was aspirated by accident, the syringe was discarded. Another clean, sterile syringe and needle were used to continue the harvest. This was pooled into a 500 ml. sterile Erlenmayer flask, partially immersed in ice water. The temperature of the cubicle during harvesting was approximately 28 C. One cardboard flat of eggs (30 eggs) was harvested at a time. The remaining dead eggs were kept at 2 C until harvested.

A suitable sample of the pooled chorio-allantoic fluid had an ELD_{50} of $10^{-9.0}$. Shortly after harvesting, the 360 ml of

choric-allantoic fluid was centrifuged for 30 minutes at 2000 rpm in an International centrifuge, Model PR-1, refrigerated at 0 C. The supernatant was transferred into sterile stainless tubes with aluminum caps. These tubes fit the No. 30 head of the Spinco Model L refrigerated centrifuge. A sample of the supernatant was tested for its virus content by both the hemagglutination test (HA) and by determining its ELD_{50}. The results of these two tests indicated no loss in virus after centrifuging the virus-containing chorio-allantoic fluid at 2000 rpm for 30 minutes.

The clear supernatant was centrifuged for 4½ hours at 31,000 rpm in the Spinco Model L centrifuge refrigerated at 2 C. During deceleration, no brakes were used. The clear supernatant was titrated for virus content with negative results. The virus was concentrated in the pellet. The pellet was grayish-white in color, approximately 1 ml in thickness and 4 mm in diameter. The supernatant was discarded. Each pellet was homogeneously resuspended into 5 ml of sterile physiological saline, made with glass-distilled water containing less than two parts per million of metal ions. The centrifuge tube was maintained partially immersed in ice water. When no visible portions of the pellet appeared in the saline suspension, this was transferred into two stainless steel centrifuge tubes. The virus suspension was centrifuged for 4½ hours at 31,000 rpm in the Spinco Model L centrifuge refrigerated at 2 C. The procedure of this centrifugation was similar to the one described above. The supernatant was tested again for virus activity with negative results. The pellet of each tube was resuspended in 9 ml of sterile physiological saline containing 0.4 percent of commercial formalin. After resuspension, the formalinized saline was left in the refrigerator for 5 days. The formalinized saline contained ND virus and constituted the antigen. The pH of the antigen was 6.3.

Each lot of antigen was titrated with a serum sample known to contain antibodies against ND virus. The best proportions of both antigen and serum were thus determined.

Procedure for performing the plate agglutination test.

Each serum sample is centrifuged at 0 C for 20 minutes at 14,000 rpm in a Model PR-1, International centrifuge. A drop of the clear supernatant is mixed on a glass plate, with a loopful of the antigen. The glass plate is maintained at room temperature. Between samples, the loop is flamed and cooled in sterile distilled water. Ten separate serum samples can be easily tested at one time. The glass plate is rotated for 2 to 3 minutes. The tests are read in a dark room by transillumination with a narrow-beam microscope light. Positive sera will show easily visible clumps which have a tendency to marginate. Negative sera do not show clumps. The rapidity of margination of the clumps varied slightly with each batch of antigen used. Six different batches of antigen were tested with essentially the same results.

Twenty-five sera from known susceptible birds were tested by the plate-agglutination test. No false positives were found. To determine the specificity of the plate test, 40 sera were tested from chickens immune to laryngotracheitis virus but susceptible to ND virus. All 40 samples were negative. Forty-five additional serum samples from chickens immune to infectious bronchitis virus but susceptible to ND virus were also tested and found negative.

One serum sample containing agglutinins against ND virus and having a hemagglutination inhibition (HI) titer of 1:80 was absorbed with the GB (Texas) strain of ND virus for 25 minutes at room temperature. After absorption, the serum did not agglutinate the antigen.

The plate agglutination test was compared with the HI test by using the sera from serial bleedings of 52 five-week-old White Leghorns vaccinated intranasally with a commercial B1 vaccine containing 75 million ELD_{50} of ND virus per ml. Results of the tests are compared in table 1. Two days before vaccination, the sera were negative for HI and agglutinating antibodies. Four days after vaccination, agglutinins were detected in the sera of two birds, but no HI antibodies were found. Fourteen serum samples collected on the 7th day were strongly positive, and 13 additional samples were considered weakly positive. In these sera, clumps were not as

large as in the strongly positive samples, and their formation not as rapid. Within the same period, only 7 sera had diagnostic HI titers—1:40 or higher. All serum samples collected between the 10th and the 17th days were definitely positive.

TABLE I

Comparison of the hemagglutination-inhibition (HI) and the plate agglutination test (PAT)

No. of serum samples	Vaccination time (days)	HI titer	PAT
52	—2	0	N
7	+4	0	5N, 2P
4	+7	0	1N, 1WP, 2P
13	+7	10	2N*, 7WP, 4P
9	+7	20	2N, 2WP, 5P
6	+7	40	1N*, 2WP, 3P
1	+7	80	1WP
72	10-17	20-1280	72P

N = negative, WP = weakly positive, P = definitely positive.
* = milky appearance of sample.

ACKNOWLEDGMENT

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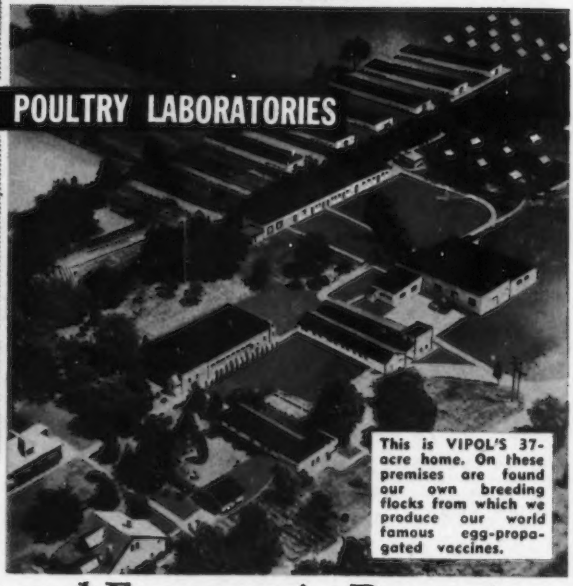

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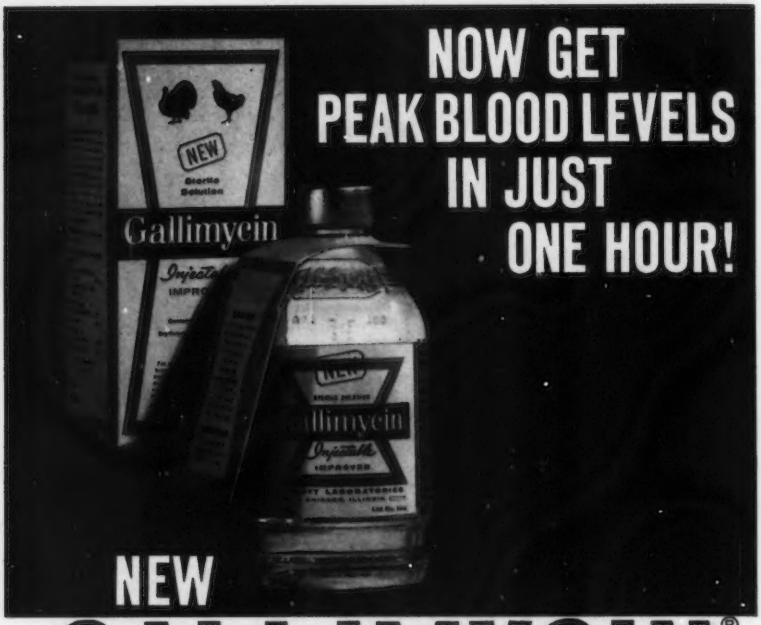

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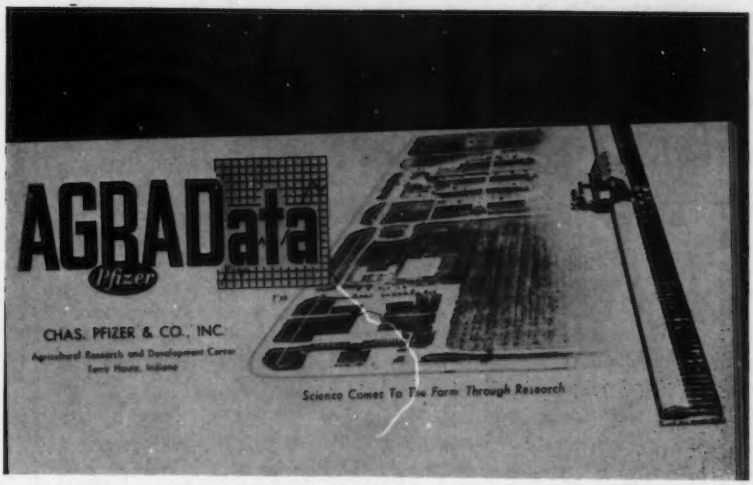

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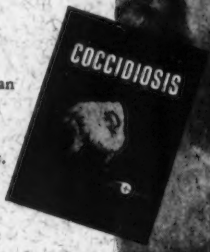

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